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Health Beneficial Effects of Carotenoids and Polyphenols from Locally Grown Fruits and Vegetables as Determined by In-Vitro Assessment of Caco-2 Cells Following Simulated Gastro-intestinal Digestion.

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List of Abbreviations:

2D-DIGE: two dimensional- differential gel electrophoresis

8-OHdG: 8-hydroxy-2' -deoxyguanosine

AAPH: 2-2'-azobis (2-methylpropionamidine) dichloride

ABTS: 2-2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

diammonium salt

$AlCl_3$: aluminium chloride

AMD: age-related macular degeneration

ARE: antioxidant response element

BCO2: β-carotene oxygenase 2

BCO1: β-carotene 15,15´-oxygenase 1

CAT: catalase

CCL2: chemokine (C-C motif) ligand 2

CD: Crohn's disease

CE: catechin equivalent

CGE: cyanidin 3-glucoside equivalents

cNOS: constitutive NOS

COX: cyclooxygenase

COX-2: cyclooxygenase 2

CTAB: cetyl trimethylammonium bromide

CVD: cardiovascular disease

CXCL: chemokine (C-X-C motif) ligand 2

DI: 2,6-dichlorophenolindophenol

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

FRAP: Ferric-reducing antioxidant power assay

GAE: gallic acid equivalents

GCL: glutamate cysteine ligase

GPx: glutathione peroxidase

GSH: glutathione

GSTs: glutathione-S-transferase

H_2SO_4: sulfuric acid

HCl: hydrochloric acid

HMGB1: high-mobility group box 1

HNO_3: nitric acid

HO-1: heme oxygenase-1

HRP: horseradish peroxidase

IBD: inflammatory bowel diseases

ICAM-1: intracellular adhesion molecule 1

IGFBP3: insulin-like growth factor binding protein 3

IKK: IκB kinase

IL: interleukin

iNOS: inducible nitric oxide synthase

Keap1: kelch-like ECH-associated protein 1

LDL: low-density lipoprotein

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

MCP-1: monocyte chemotactic protein 1

MDA: malondialdehyde

MTBE: methyl tert-butyl ether

Na_2CO_3 : sodium carbonate

NAD(P)H: nicotinamide adenine dinucleotide phosphate

$NaNO_2$: sodium nitrite

NaOH: sodiumhydroxide

NEMO: NF-κB essential modulator

NF-κB: nuclear factor kappa B

NO: nitric oxide

NOS: nitric oxide synthase

NOX-2/4: NAD(P)H oxidase

NQO1: NAD(P)H quinone oxidoreductase 1

Nrf2: nuclear factor erythroid 2- related factor 2

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffer saline

PET: polyethylene

PGE-2: prostaglandin E2

PGF2a: prostaglandin F2a

RA: retinoic acid

RAR: retinoic acid receptor

ROS: reactive oxygen species

SOD: superoxide dismutase

STAT: signal transducers and activators of transcription

THF: tetrahydrofuran

TMB: tetramethylbenzidine

TNF-α: tumor necrosis factor alpha

UC: ulcerative colitis

VCAM-1: vascular cell adhesion protein 1

VCE: vitamin C equivalents

VCEAC: vitamin C equivalent antioxidant capacity

WHO: World Health Organization.

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Summary:

Fruit and vegetable consumption has been promoted by the World Health Organization (WHO) and other health associations worldwide, and many epidemiological studies have shown that the intake of fruits and vegetables is correlated with the prevention of chronic diseases. Carotenoids and polyphenols (Fig 1 and Fig 2) are the most important lipid- and water soluble phytochemicals in fruits and vegetables, respectively, and in intervention trials and during *in vitro* studies, they have shown anti-inflammatory and anti-oxidative properties, which are regarded to contribute to the observed reduction of chronic diseases, including cardio- metabolic complications, and several types of cancer, but also additional ones, such as inflammatory bowel disease (IBD).

Inflammatory bowel disease is an umbrella term for a group of inflammatory disorders, the most well-known being ulcerative colitis (UC) and Crohn´s disease (CD). In ulcerative colitis, the colon is inflamed while in Crohn´s disease any part of the gastro-intestinal tract can be affected. Both phytochemical groups have been hypothesized to have some positive impact on the gut mucosa, strengthening gut barrier properties, while lowering oxidative stress and inflammation related processes. However, very little data is available on the potential to employ frequently consumed fruits and vegetables rich in carotenoids and polyphenols as a preventive or adjuvant strategy in IBD.

In a pilot study, we studied first the impact of beta-carotene and lycopene under the form of micelles on inflammatory stimulated

Caco-2 cells. The results showed that single carotenoids had no statistical significant anti-inflammatory effect on selected biomarkers, among others a key transcription factor, *i.e.* NF-κB and two end products, *i.e*. IL-8, and NO.

Fig 1: Chemical structure of some major carotenoids [1].

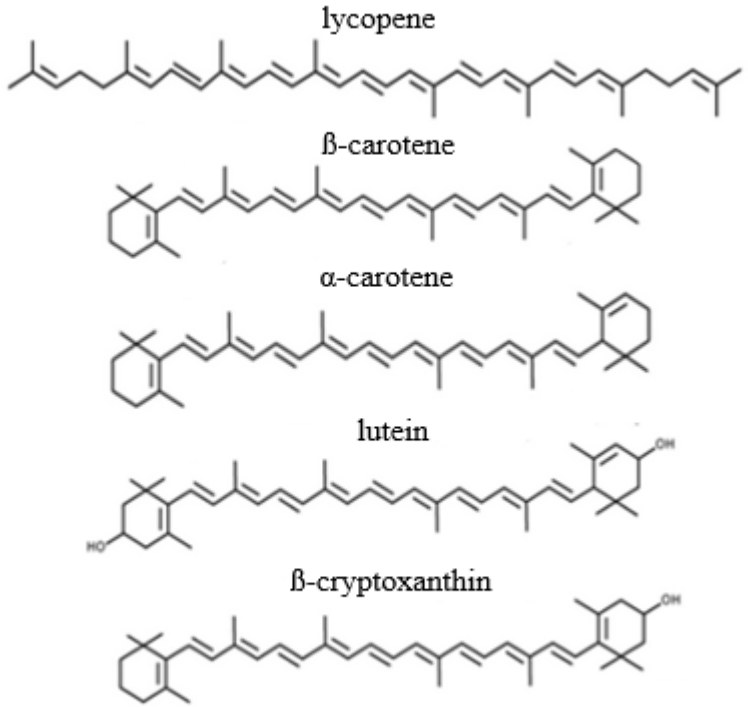
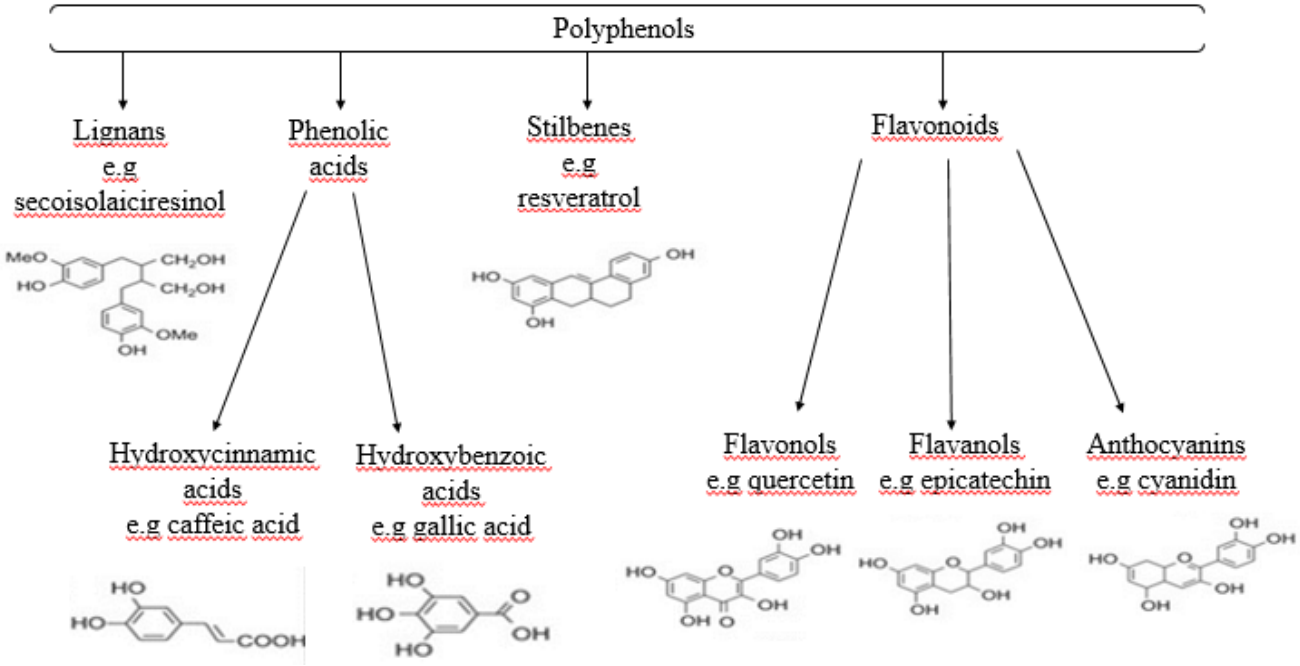


Fig.2 :Chemical structure of some major polyphenols [2].



In the present investigation, we therefore strived to investigate firstly the changes from native occurrence to the bioaccessible form of carotenoids and polyphenols from frequently consumed, in Luxembourg, plums and cabbages in Luxembourg, during gastro-intestinal digestion, as both carotenoids and polyphenols may undergo substantial changes following their intake, and only a small fraction may finally be available to the body.

Therefore, we investigated 27 cabbage and 17 plum varieties and their carotenoid and polyphenol content, as well as their micro- and macronutrients. In this part of the study we strived to find the varieties that were richest in carotenoids and polyphenols, and also to show which ingredients were related to their antioxidant potential, as assessed *in vitro*.

In the second part, we then submitted the selected varieties, *i.e*. cabbage (Duchy (+/-) , Scots Kale (-/+), Kale (+/+) and Kalorama (-/-) and plum varieties (Cherry plum (+/-), Plum 620 (-/+), Italian plum (+/+), Ersinger (-/-)) with contrasting carotenoid and polyphenol content (+ indicating rich, - indicating low content) as well as varieties high or low in both to *in vitro* gastro-intestinal digestion. Within this part of the study, we wanted to improve our understanding regarding changes of the carotenoids and polyphenol profile during digestion, but also following different common kitchen procedures such as freezing, thawing, and various heating methods.

Finally, by employing these digesta on a Caco-2 cell model and a coculture of Caco-2/HT-29 MTX cells, we also intended to investigate how absorbable the carotenoids and polyphenols were in

this model, and whether the typically overlooked mucus layer, produced by the HT-29 MTX cells, resembling better physiological circumstances, influenced cellular uptake.

In a third part of the study, we investigated the effects of carotenoids and polyphenols on stimulated intestinal inflammation, including selected inflammatory markers, intracellular signalling cascades, as well as their impact on proteomic expression via the help of a Caco-2 cell model and a novel developed triple culture, composed of the coculture Caco-2/HT-29 and THP-1 (macrophage-like) cells, more closely resembling the situation of an inflamed gut with infiltrating immune cells.

First, it was shown that the carotenoid and polyphenol profile of the tested varieties varied a lot, from 55 µg/100g – 13 mg/100g for carotenoids and 95 µg/100g – 27 mg/100g for polyphenols in cabbage and from 247 µg/100g – 2 mg/100g for carotenoids and 14 mg/100g – 151 mg/100g for polyphenols in plums. We also investigated which molecules could best predict antioxidant capacity by employing multiple linear regression models. For cabbage, lutein, ascorbic acids and phenolic compounds were the best predictors for antioxidant capacity and for plums selenium, total sugars, ascorbic acid and phenolic compounds. Results also demonstrated that the *in vitro* - gastrointestinal digestion as well as the investigated kitchen-procedures did not significantly impact carotenoids but that the polyphenols were significantly reduced in concentration, by up to – 89% or -51 %, mostly by chop/boiling and chop/steaming.

Then, we observed that carotenoid bioaccessibility did not significantly differ between cabbage and plum varieties. The results showed also that xanthophylls were better bioaccessible than carotenes. The polyphenol bioaccessibility was low (<10%), probably due to the addition of cream to our test meal. The uptake of the carotenoids differed substantially between the varieties (0.3-4.1 % ,or, in absolut amounts, between 0.2 – 9.1 µg (Table 1)), but was also not significantly different between cabbages and plums. In contrast to the bioaccessibility, the cellular uptake of the carotenes was higher than the uptake of xanthophylls. The results showed also that the coculture had a slightly but significant higher carotenoid uptake than the monoculture (2,59 % vs. 0,99 %).

Table 1: Carotenoid uptake in the Monoculture and Coculture following gastro-intestinal digestion of different cabbage and plum varieties.

Variety	**Monoculture Total carotenoid uptake in µg**	**Coculture Total carotenoid uptake in µg**
Duchy	3	9.1
Scots Kale	0.2	4.4
Kale	3.2	1.3
Kalorama	0.2	0.4
Cherry plum	0.2	0.5
Plum 620	0.2	0.2
Ersinger	0.2	0.5
Italian plum	0.5	1.4

Finally, the study showed that some of the cabbage and plum varieties had some impact on the selected inflammatory markers, intracellular signalling cascades and proteomic response. The results

showed that IL-8 was decreased by all the plum varieties (Cherry plum, Plum 620, Ersinger, Italian plum) in both cell cultures. Scots Kale and Kalorama decreased Il-6 secretion and NF-κB activation in the THP-1 cells. Ersinger and Italian plum decreased Nrf-2 mRNA expression and decreased Nrf-2 nucleus accumulation in the coculture, emphasizing that some anti-inflammatory pathways were influenced in a positive, i.e. anti-inflammatory way. The proteomic analysis showed that in the monoculture, 68 protein spots were significantly differentially expressed due to the Kale and Italian plum treatment. From these, 27 proteins were uniquely identifiable proteins (not counting the isoforms). In the coculture, 206 protein spots were significantly differentially expressed; from these 76 proteins were uniquely identifiable proteins (not counting the isoforms).

All the results emphasized that the cabbage and plum varieties selected had some anti-oxidant and ant-inflammatory potential in our models, highlighting that cabbage and plum may constitute food items positively influencing inflammation in IBD. However, the results also showed that the effects were not well correlated to the carotenoid and polyphenol profile, suggesting the presence of other health beneficial compounds (i.e ascorbic acid, vitamin E, glucosinolates, dietary fibers), or unknown synergistic effects between phytochemicals and the food matrix. More research toward alleviating IBD by dietary intervention with fruits and vegetables rich in phytochemicals is warranted.

Reference List

1. J. Fiedor and K. Burda, Potential role of carotenoids as antioxidants in human health and disease, *Nutrients*, 2014, **6**, 466-488.
2. J. Bensalem, A. Dal-Pan, E. Gillard*, et al.*, Protective effects of berry polyphenols against age-related cognitive impairment, *Nutrition and Aging*, 2015, **3**, 89-106.

1. Pilot Study (Chapter 1):

This Chapter 1 corresponds to our hypothesis building. We investigated whether lycopene or beta-carotene in micelles might have an impact on selected inflammatory markers and on the proteome of inflamed Caco-2 cells. The results of this work have been published in the British Journal of Nutrition: Kaulmann, A., Serchi, T., Renaut, J., Hoffmann, L. and Bohn, T. (2012) 'Carotenoid exposure of Caco-2 intestinal epithelial cells did not affect selected inflammatory markers but altered their proteomic response', *British Journal of Nutrition*, 108(6), pp. 963–973

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Carotenoid Exposure of Caco-2 Intestinal Epithelium Cells did not Impact Selected Inflammatory Markers but Altered Their Proteomic Response

Abstract

Carotenoid consumption has been linked to a number of beneficial health effects, including the reduction of chronic diseases such as cancer and cardiovascular complications. However, no data are available on their action on the intestinal epithelium, being exposed to the highest concentrations of carotenoids in the human body, and where they could act preventively on intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis. The objective of the present study was to investigate whether lycopene and β-carotene in micelles (M), at concentrations that could be reached via the diet (10–25 µg/ml) could aid in the reduction of TNF-α plus IL-1β-induced inflammation of Caco-2 human epithelial cells. The impact on biomarkers of inflammation, including IL-8, NO and cyclo-oxygenase-2 (through PGE-2α), and the NF-κB and mitogen-activated protein kinase (MAPK) pathways of intracellular signalling cascades were evaluated compared with controls (empty M). Furthermore, proteomic analyses were conducted from total cellular protein extracts. The results revealed that isolated carotenoids had no statistical significant anti-inflammatory effect on the biomarkers observed, or on the regulation of NF-κB and MAPK. Nevertheless, analyses of the proteome suggested that fifteen proteins were significantly ($P < 0.05$, expression ratio > 1.3) differentially regulated following β-carotene exposure, participating mostly in metabolic activities including antioxidant mechanisms, such as glutathione S-transferase A1. Only one protein was differentially regulated by lycopene (profilin-1). To our knowledge, this is the first attempt to

investigate pathways involved in the action of carotenoids on the intestinal epithelium.

1.1 Introduction:

Carotenoids are lipid-soluble, C-40-based pigments that can be synthesized by vegetables and fruits and certain fungi, but not by animals including humans. Of the approximately 700 identified carotenoids, only about fifty are of importance in the human diet (1). In addition to the well-known provitamin A activity of several carotenoids, it has been suggested that carotenoids have further beneficial effects, including the prevention of cancer (2,3), CVD (4,5) and diabetes (6,7). Carotenoids can act as antioxidants with the potential to remove free radicals, either by a direct reaction with radicals, resulting in the formation of harmless products, or by disrupting radical chain reactions, avoiding further damage of cellular compounds, such as membrane lipids. There exists a growing awareness that oxidative stress and the resulting inflammation play an important role in the development of several chronic diseases (8), including cancer (9), diabetes (6) and inflammatory bowel diseases (IBD) (10). It has been shown that reactive oxygen species can activate several signal transduction pathways such as the mitogen-activated protein kinase (MAPK) signalling cascade, as well as amplifying the activation of the nuclear transcription factor NF-κB (11), both resulting in enhanced formation of pro-inflammatory mediators such as cytokines (12).

Ulcerative colitis (UC) and Crohn's disease (CD) are the most predominant IBD, affecting over 2.2 million people of the European population, with a continuous increase over the past decades (www.efcca.org), with reasons remaining speculative, but including

increased hygienic conditions and decreased immune stimulation (13). Pathological complications of IBD may result following redox-mediated dysregulation of signalling cascades and/or gene expression (11). Major intracellular signalling cascades that are responsible for the production of inflammatory molecules include NF-κB and MAPK, modulating action via their regulation and induction of target genes (14). The resulting inflammation of the small (mainly CD) and large (both CD and UC) intestinal epithelium is chronic, has a low remission rate to medical treatment such as corticosteroids or non-steroidal anti-inflammatory agents (15,16), and may develop into cancer (15,16). Thus, the search for preventive strategies targeting the onset of inflammation in the gut is paramount. The implication of carotenoids in inflammatory processes in the human body is only marginally understood. It was noted that patients affected by early-stage IBD had low serum concentrations of micronutrients including Se and β-carotene (17), with similar results for several carotenoids in CD (10,18) and UC and CD subjects (19). On the other hand, markers of oxidative stress or inflammation including TNF-α, IL-1β and lipid peroxidation were increased in adult subjects with CD (20,21), as was oxidative DNA damage (22). Also increased NO was found as a mediator of inflammation in subjects with UC and CD (23,24), and was present in higher concentration in intestinal epithelial cells of IBD patients. Thus, it could be hypothesised that there exists a relationship between low antioxidant status and increased inflammation. However, carotenoid absorption is typically low (1) and carotenoid concentration decreases from the food matrix to the tissues. As the

concentration of carotenoids is highest in the intestine, it is possible that their effect in this tissue is comparatively pronounced.

In the present study, the aim was to investigate the potential of carotenoids to interfere with inflammatory processes in the gut epithelium. For this purpose, differentiated human intestinal Caco-2 cells stimulated for inflammation with TNF-α and IL-1β were exposed to lycopene and β-carotene emulsified in artificial micelles, and inflammatory markers including IL-8, NO, cyclo-oxygenase-2 (COX-2) activity via PGE-2α measurement, as well as the signalling molecules NF-κB and MAPK were investigated. In addition, a proteomic study on extracted proteins from the whole cellular lysate following carotenoid exposure was performed using two-dimensional differential in-gel electrophoresis (2D-DIGE).

1.2 Materials and methods

1.2.1 Chemicals

All products were of analytical grade or higher. Unless otherwise stated, all chemicals including oleic acid, monoolein, L-α-phosphatidylcholine, IL-8, lipopolysaccharide, TNF-α, sodium taurocholate hydrate, resazurine, arachidonic acid, penicillin/streptomycin mixture and non-essential amino acids were procured from Sigma-Aldrich. High-purity (18 mΩ) water was prepared with a purification system from Millipore and used throughout. Lycopene was purchased from Extrasynthése and β-carotene was purchased from Dr Ehrenstorfer GmbH.

1.2.2 Cell culture

The TC-7 subclone (ATCC no. HTB-37) of the Caco-2 parental cell line was derived from a tumour isolated by J. Fogh (25) and was a generous gift from Monique Rousset (Nancy University). Cultures were routinely maintained in 75 cm2 plastic flasks (Nunclone; Nunc) at 37°C and 10 % CO2 in a CB-210 CO2 incubator (Binder GmbH). Cells were grown in Dulbecco's modified Eagle's medium (DMEM + GlutaMAX™; Gibco), supplemented with 1 % non-essential amino acids, 20 % heat-inactivated fetal bovine serum (Gibco), 1 % of a mixture of penicillin and streptomycin (10000 units and 10mg/ml, respectively) and subcultured weekly after reaching an approximately 80 % confluence. For all experiments, 1-week differentiated cells were used (passage 50-81). Transepithelial electrical resistance, a valid method for determining epithelial tightness and cellular differentiation, was used to evaluate the differentiation status of the cells, and no statistical significant differences were found compared with 2 weeks of differentiation.

1.2.3 Preparation of artificial micelles

For the preparation of artificial micelles, the original protocol of Biehler *et al.* (26) was adapted. In short, for the preparation of the medium, 0·11g sodium taurocholate were weighed into a 100ml glass vial, and 100ml DMEM+glutamax (20 % fetal bovine serum, 1 % non-essential amino acids and 1 % penicillin and streptomycin) were added under a laminar flow box. In parallel, 12.8 mg L-α-phosphatidylcholine, 18.0 mg monoolein and 1.0 mg oleic acid were weighed on a six-digit balance (Metler Toledo) into another 100 ml glass vial. The vial was enwrapped with aluminium foil and kept on

ice. Next, 4.0 mg of either lycopene or β-carotene were dissolved in 8 ml chloroform. Then, 2 ml (for 10 mg/l) or 5 ml (for 25 mg/ l) of these solutions were added into the enwrapped 100 ml glass vial and the organic solvent was removed under a stream of N_2 during 5 min. Finally, 100 ml of the prepared medium described above were added and the mixture sonicated for a total of 30 min. After sonication, the solution appeared clear and no unsolubilised residues were visible. The last step consisted of a sterile filtration under a laminar flow box through a 0.22 µm filter (PALL Life Sciences). The mixture was then divided into aliquots and stored at -80 C until usage. In parallel, micelles without lycopene and β-carotene were prepared as negative controls.

1.2.4 Cell viability

To test the viability of the cells, especially following exposure to artificial micelles, the resazurine assay was used. Resazurine is a water-soluble dye, which can pass through cellular membranes. In the mitochondria, the oxidised blue resazurine is then reduced to the highly fluorescent pink compound resorufin, its amount being directly proportional to cell viability. Cells were exposed for 24 h to artificially produced micelles with or without carotenoids, and to pro-inflammatory cytokines used to induce inflammation (100 ng TNF-α/ml together with 25 ng IL-1β/ml). After the treatment, cells were washed with basal medium (DMEM+GlutaMAX™, supplemented with 1 % non-essential amino acids, 20 % heat inactivated fetal bovine serum, 1 % of a mixture of penicillin (10000 units) and streptomycin (10 mg/ml), respectively) and incubated for 2 h with a medium containing 400 µM-resazurine in the dark (37°C, 90 % air, 10 %

CO2). Fluorescence was measured in a BioTek Synergy 2 plate reader, with excitation at 530 nm (530 (25) nm filter) and emission recorded at 590 nm (590 (25) nm filter). Cell viability is expressed as mean cell viability (in %) compared with cells without treatment (set at 100%).

1.2.5 Exposure experiments for studying IL-8, NO and PGE-2α, mitogen-activated protein kinase and NF-κB

Cells were grown on twenty-four-well plates (BD Biosciences) and six different treatments were performed (Table 1). To each well, a total volume of 10 µl TNF-α (10 µg/ml) and 10 µl IL-1β (25 µg/ml) was added. Each experiment was performed on three different days (except for NF-κB and MAPK on 4 different days), with each individual treatment done as duplicates (IL-8, PGE-2α and NO). Secretion of IL-8, PGE-2 and NO is expressed as percentage of the positive control (blank (B) + micelles (M) + stimulus (S)). In order to verify uptake of the carotenoids by cells, Caco-2 cells grown on six-well plates (BD Biosciences) and incubated for 4 h at 37°C with 2 ml of the prepared medium/micelle mixture, containing either lycopene (10 or 25 µg/ml) or β-carotene (10 or 25 µg/ml), were extracted and analysed as described earlier (26).

Table 1 :

Exposure of 1-week differentiated Caco-2 epithelial cells to various carotenoid treatments (n 6 per treatment).

Exposure group	**Treatment of cells**
Blank	Untreated: no inflammatory stimuli or micelles
Blank + micelles (negative control)	24 h with empty micelles
Blank, micelles + stimuli (positive control)	TNF-α, IL1-β* (28 h) and empty micelles (4-28h)
Blank, micelles, stimuli + all-*trans*-lycopene (10 or 25 μg/ml)	TNF-α, IL1-β (28 h) and micelles containing 10 or 25 μg/ml lycopene (4-28h)
Blank, micelles ,stimuli + all-*trans* β-carotene (10 or 25 μg/ml)	TNF-α, IL1-β (28 h) and micelles containing 10 or 25 μg/ml β-carotene (4-28h)

*: 25 ng/ml IL-1β and 100 ng/ml TNF-α simultaneously

1.2.6 Determination of IL-8 secretion

IL-8 concentration in the supernatant was measured by an enzyme immunoassay kit from BD OptEIA (Becton Dickinson), according to the manufacturer's recommendation. In short, 50 µl ELISA diluent and 100 µl of the supernatant were added to a ninety-six-well plate, which was pre-coated with a monoclonal antibody for human IL-8. The plate was incubated for 2 h and washed before the addition of the monoclonal IL-8 antibody and streptavidin-horseradish peroxidase. The plate was again incubated for 1 h, washed with washing buffer and 100 µl substrate of horseradish peroxidase (tetramethylbenzidine; 3,3´,5,5´-tetramethylbenzidine) was added. Finally, following another incubation of 30 min, stop solution (1M-phosphoric acid) was added and absorbance was read at 450 nm in a BioTek Synergy 2 plate reader. IL-8 concentration (pg/ml) was calculated using an external IL-8 standard curve.

1.2.7 Determination of cyclo-oxygenase-2 activity

Cells were treated as described above; however, to induce COX-2 activity, cells were additionally incubated for 10min with arachidonic acid (10mM in PBS). Concentration of PGE-2α in the supernatant was determined using the PGE-2 enzyme immunoassay kit from Cayman Chemical following the manufacturer's instructions. Briefly, 50 µl of culture medium, PGE-2-acetylcholinesterase conjugate and PGE-2α monoclonal antibodies were added to a ninety-six-well plate supplied with the kit. This goat polyclonal anti-mouse IgG antibody pre-coated plate was incubated for 18 h at 4°C. The plate was then washed to remove unbound reagents and 200 µl reagent (substrate for the acetylcholinesterase) was added. The plate was again incubated for

90min at room temperature and absorbance read at 412 nm in a BioTek Synergy 2 plate reader. The concentration of PGE-2α (pg/ml) was determined by external calibration curves.

1.2.8 Determination of NO secretion

NO is unstable and is rapidly oxidised into nitrate (NO_3^-) and nitrite (NO_2^-). The measurement of NO (as NO_3^- and NO_2^-) was carried out by the Nitric Oxide Assay Kit from Calbiochem (Merck KGaA) and was based on the Griess assay (40). The Griess reagent, however, does not measure nitrate, therefore the NADPH-dependent enzyme, nitrate reductase, was used to convert the nitrate to nitrite before quantification. Briefly, 85 µl of culture medium were added to a ninety-six-well plate. Then nitrate reductase (10 µl) and NADH (10 µl) were added to the wells. The plate was incubated for 20 min at room temperature. Next, the Griess reagent (100 µl) was added and the plate was shaken for 5min before reading absorbance at 540 nm in a BioTek Synergy 2 plate reader. The concentration of NO (mM) present in the samples was calculated by means of an external standard curve.

1.2.9 Determination of NF-κB

Cells were grown in 75cm^2 plastic flasks (Nunclone; Nunc) and five different treatments (with n 4 replicas each) were performed. Cells were treated as described above; only lycopene at 10 µg/ml was omitted due to reagent limitations. The nuclear extract was obtained from the cultured cells by using the Cayman Nuclear Extraction kit (Cayman Chemical). Briefly, 10^7 cells were collected and centrifuged at 300 g for 5min at 4°C. The supernatant was discarded and the cells were resuspended in PBS containing phosphatase inhibitors, and

centrifuged again. This step was repeated before the addition of hypotonic buffer (500 µl). The cells were then incubated on ice for 15 min, before detergent addition (10 % Nonidet P-40 Assay Reagent, 50 µl) and centrifuged for 30s at 4°C. The supernatant containing the cytosolic fraction was transferred to a 1.5 ml microcentrifuge tube and stored at -80°C until analysis. Pellets were resuspended in 50 µl nuclear extraction buffer (containing protease and phosphatase inhibitors and dithiothreitol) and incubated on ice (15 min). This step was repeated before centrifugation at 14000 g (10 min, 4°C). The supernatant was transferred to a 1.5 ml microcentrifuge tube and was either further examined or stored at -80°C until analysis. The concentration of NF-κB was measured by the NF-κB (p65) ELISA kit (Cayman Chemical) following the manufacturer's protocol. In short, 90µl of complete transcription factor buffer and 10 µl of the nuclear extract were added to a ninety-six-well plate and incubated overnight at 4°C. The plate was pre-coated with a consensus double-stranded DNA sequence containing the NF-κB response element. After incubation, the plate was washed five times with 200 µl wash buffer, and the primary antibody added. The plate was covered and incubated for 1h at room temperature. Following incubation, the plate was again washed with wash buffer and a second antibody conjugated to horseradish peroxidase was added. The plate was covered and incubated for 1h at room temperature. After incubation, the plate was washed with 200 µl wash buffer, and 100 µl developer solution was added. The colorimetric development was monitored between 15 and 45 min, and 100 µl stop solution was added. The absorbance was read at a wavelength of 450 nm in a BioTek Synergy 2 plate reader.

1.2.10 Determination of mitogen-activated protein kinase activation

Cells were grown and exposed as described for NF-κB (with n 4 replications for each of the five exposures). The nuclear extract was obtained by following the cell extraction protocol of the p38 MAPK Immunoassay Kit (Invitrogen). The cells were collected in PBS by scraping them from the culture flasks. Then, two washing steps with PBS followed by centrifugation for 1 min at 16500 g were performed and the supernatant was discarded. The cell pellet was lysed in cell extraction buffer for 30 min on ice and was vortexed every 10 min. The extract was then transferred to a 1.5 ml microcentrifuge tube and centrifuged for 10 min at 18000 g at 4°C. The lysate was divided into aliquots into 1.5 ml microcentrifuge tubes. The nuclear extracts (100 µl) were added to anti-p38 antibody pre-coated ninety-six-well plates. After 2 h of incubation, the wells were washed with wash buffer (200 µl) and 100 µl anti-p38 MAPK antibody was added. The plate was incubated for another 1h and after an additional washing step with 200 µl wash buffer, 100 µl horseradish peroxidase-labelled anti-rabbit IgG were added. After a third incubation and washing step with 200 µl wash buffer, a chromogen (tetramethylbenzidine) was added and 30 min later, the reaction was stopped by adding 100 µl stop solution and the absorbance read at 450 nm in a BioTek Synergy 2 plate reader. The concentration of MAPK (pg/ml) present in the samples was calculated by external calibration curves.

1.2.11 Proteomic analyses following carotenoid exposure of Caco-2 cells.

For 2D-DIGE, an earlier protocol (27) was adapted. Unless otherwise stated, materials were from GE Healthcare. For each treatment (B, B+M, B+M+S, lycopene 10 µg/ml and β-carotene 10 µg/ml or 25 µg/ml), four TC175 flasks (Nunclone; Nunc) were used to obtain sufficient biological cellular material. The total protein extract from the Caco-2 cells was obtained by using the extraction protocol of the p38 MAPK immunoassay kit from Invitrogen. The protein concentration was determined by the Bradford method and a 1mg/ml bovine serum albumin standard (28). The pH of the extracted protein samples was adjusted to 8.5 with 3M-Tris, and each extract was labelled by the minimal labelling process, following the manufacturer's instructions. Briefly, 30 µg of proteins were labelled with either 240 pmol Cy3 or Cy5 protein labelling dye, respectively. In addition, an internal standard was created by pooling aliquots of all protein extracts obtained, and 30 µg labelled with Cy2 dye. For the labelling, 240 pmol of dye were added to each tube, briefly centrifuged and vortexed and incubated for 30 min on ice in the dark. To stop the labelling reaction, 1 µl of 10mM-lysine was added; samples were briefly centrifuged, vortexed and incubated for 10 min on ice in the dark. Afterwards, the Cy3- and Cy5 labelled extracts were combined with the Cy2-labelled internal standard (Table 2).

Table 2:

Experimental design for the proteomic experiment employing two-dimensional differential in-gel electrophoresis following six different carotenoid treatments (n 4 replica each, no. 1-4)*.

Gel No	Cy2	Cy3	Cy5
1	Pooled internal standard	Blank no. 1†	β-Carotene 25 μg/ml no. 3‡
2	Pooled internal standard	Blank no. 2†	β-Carotene 25 μg/ml no. 4‡
3	Pooled internal standard	Blank/micelles no. 1§	β-Carotene 10 μg/ml no. 3‡
4	Pooled internal standard	Blank/micelles no. 2§	β-Carotene 10 μg/ml no. 4‡
5	Pooled internal standard	Blank/micelles/stimulus no. 1‖	Lycopene 10 μg/ml no. 3¶
6	Pooled internal standard	Blank/micelles/stimulus no. 2‖	Lycopene 10 μg/ml no. 4¶
7	Pooled internal standard	Lycopene 10 μg/ml no. 1¶	Blank/micelles/stimulus no. 3‖
8	Pooled internal standard	Lycopene 10 μg/ml no. 2¶	Blank/micelles/stimulus no. 4‖
9	Pooled internal standard	β-Carotene 10 μg/ml no. 1‡	Blank/micelles no. 3§
10	Pooled internal standard	β-Carotene 10 μg/ml no. 2‡	Blank/micelles no. 4§
11	Pooled internal standard	β-Carotene 25 μg/ml no. 1‡	Blank no. 3†
12	Pooled internal standard	β-Carotene 25 μg/ml no. 2‡	Blank no. 4†

* Each cellular protein extract obtained following exposure (30 μg) was labelled with one of the three fluorophores (Cy2, Cy3 or Cy5). The pooled internal standard contained equal amounts of protein extracts from each treatment and was labelled with Cy2, the other samples were labelled with Cy3 or Cy5.
† Without stimuli and micelles.
‡ β-Carotene (10 or 25 μg/ml), cells stimulated with TNF-α/IL1-β mixture (28 h) exposed to micelles containing either 10 or 25 μg β-carotene/ml (24 h).
§ Without stimuli but with empty micelles (negative control).
‖ With stimuli and empty micelles (positive control), *i.e*. TNF-α plus IL1-β at 100 and 25 ng/ml (28 h) and exposed to empty micelles for 24 h.
¶ Lycopene (10 μg/ml), cells stimulated with TNF-α/IL1-β mixture (28 h) exposed to micelles containing 10 μg lycopene/ml (24 h).

The volume was adjusted to 450 µl with sample buffer (urea (7M), thio-urea (2M), (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (0·5%) and bromophenol blue (traces)), and then 9 µl of biolyte pH 3–10 ampholyte buffer (Bio-Rad, Nazareth-Eke, Belgium) and 2.7 µl of destreak reagent were added. A total of 90 µg proteins (30 µg per sample and 30 µg of internal sample) were thus loaded on each strip and 2D-DIGE was performed. The use of an internal standard reduced the possibility of erroneous conclusions due to different concentration loads or differences between conditions of each strip or gel. The samples were added on an Immobiline DryStrip (24cm, pH 3–10 non-linear; BioRad) and incubated overnight at room temperature to achieve optimal passive rehydration of the strip and sample loading. Proteins were then subjected to isoelectric focusing on an IPGphore III at 20°C. Mineral oil was added on the strips to prevent evaporation. The voltage was stepwise increased from 30 to 10000 V during the first 21 h and then stabilised at 10000 V for 8 h (about 120 kVh of the total current applied in total to each strip). Following the first dimension separation, strips were equilibrated for 15 min in equilibration buffer (2D Gel DALT; Gelcompany) containing also urea and dithiothreitol, and then for another 15 min in the same buffer containing iodoacetamide instead of dithiothreitol. After equilibration, strips were loaded on precast gels (2D gel DALT NF 12.5%; Gelcompany) for second dimensional separation, carried out on an Ettan DALT II system with 0.5 W/gel for 2 h and then 2.5 W/gel for 14 h at 25°C. The gels were scanned using a Typhoon 9400 scanner (Molecular Dynamics Inc.) and analysed by the DeCyder 2D Differential Analysis version 7.0. The protein images were produced

by excitation of gels at 488, 532 and 633 nm (Cy2, Cy3 and Cy5, respectively) and emission at 520, 610 and 670 nm (Cy2, Cy3 and Cy5, respectively) using the Typhoon 9400 scanner at a spatial resolution of 100 µm. Selected spots of interest (abundance of variation 1.3-fold, $P< 0.05$) were located on a gel and a 'picking list' was generated. The spot picking, digestion and the spotting of the samples on matrix-assisted laser desorption/ionisation (MALDI) disposable targets plates (4800; Applied Biosystems) were done automatically using the Ettan Spot Handling Workstation. Peptide mass fingerprint and MS/MS analyses were carried out using the 4800 Proteomics Analyser (Applied Biosystems). Calibration was done with the peptide mass calibration kit 4700 (Applied Biosystems). Proteins were identified by the SWISSPROT database (version 20100924 with 519538 sequences) with 'Homo sapiens' as taxonomy, using GPS Explorer Software version 3.6 (Applied Biosystems) including MASCOT (Matrix Science, www.matrixscience.com). All searches were carried out allowing for a mass window of 150 ppm for the precursor mass and 0.75 Da for fragment ion masses. The search parameters allowed for carboxyamidomethylation of cysteine as fixed modification. Oxidation of methionine and oxidation of tryptophan (single oxidation, double oxidation and kynurenin) were set as variable modifications. Proteins with probability-based MOlecular Weight SEarch (MOWSE) scores ($P<0.01$) were considered as positively identified.

1.2.12 Statistical analysis

Original data were verified for normality by Q–Q plots and checked for equality of variance by boxplots. Wherever required, data were log-transformed for statistical evaluation. Univariate models were then created with the observed parameters (e.g. IL-8 concentration) as the dependent variable and the different carotenoids and their concentrations (also including the controls) as fixed factors. Following significant Fisher's F tests, individual post hoc tests were carried out to determine differences compared with the control (Dunnet's test). A P value below 0.05 (two-sided) was chosen to indicate significance. Unless otherwise stated, all data are expressed as mean percentages (compared with the positive control) and standard deviations.

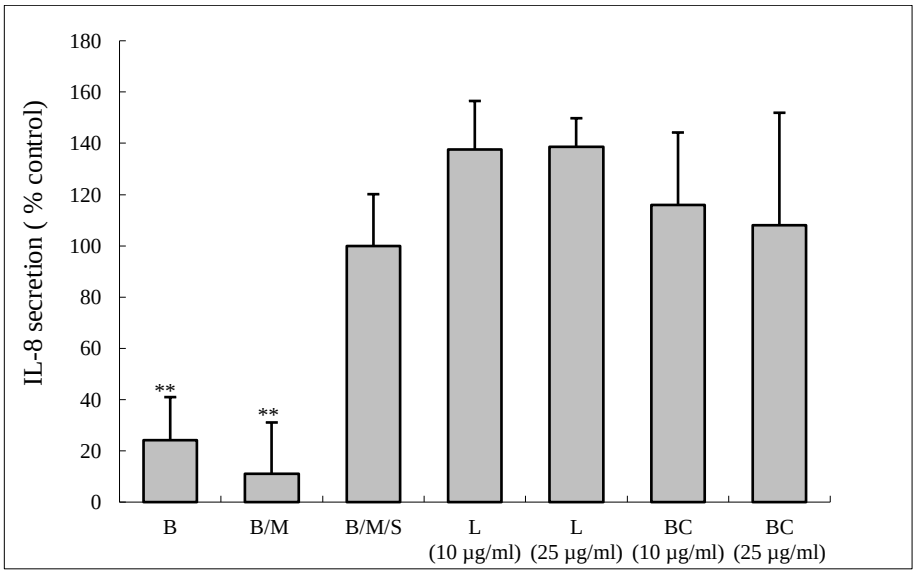
1.3 Results

1.3.1 Cell viability, IL-8, PGE-2 and NO secretion following carotenoid exposure

The different carotenoid treatments (Table 1) did not result in a significant reduction of Caco-2 cell viability. Furthermore, treatment with micelles per se (B/M) and with pro-inflammatory cytokines (B/M/S) did not result in significant changes of cell viability (Table 3). Mean uptake of β-carotene into cells was approximately 7.2 %, and that of lycopene 4.3 %. The addition of TNF-α and IL-1β resulted in a 4.1-fold induction of the IL-8 secretion compared with the negative control (Fig. 1). Neither empty micelles (B/M) nor the addition of lycopene or β-carotene (10 and 25 µg/ml) to the Caco-2 cells did result in any significant effect on the IL-8 secretion compared with the positive controls (Table 3). PGE-2 production was already high in

unstimulated cells (B and B/M) compared with the positive control. The addition of TNF-α and IL-1β did not result in any further induction of COX-2 activity. The carotenoid treatment likewise did not show any significant effect (Table 3). Similarly, the NO secretion in unstimulated cells (B and B/M) was already high compared with the positive control. The addition of the pro-inflammatory cytokines and the carotenoid treatment had no further significant impact on NO secretion (Table 3).

Figure 1:



Effect of carotenoids, lycopene and β-carotene, on the IL-8 secretion of Caco-2 cells stimulated for inflammation with TNF- α (100 ng/ml) and IL-1β (25 ng/ml) for 24 h. Cells were exposed to various treatments and the IL-8 secretion was measured after 28 h. Values are means from six independent replicates compared with the control (blank (B)/micelles (M)/stimulus (S) = 100 % (n=12 replicates)), with standard deviations represented by vertical bars. B/M, B+M; B/M/S, B+M+S; L, lycopene, BC, β -carotene, **Mean values were significantly different by Dunnet's test ($P < 0.01$).

1.3.2 NF-κB and mitogen-activated protein kinase

The influence of the two different carotenoids was also investigated on intracellular signalling cascades via NF-κB and MAPK activation. TNF-α and IL-1β induced the NF-κB activity 3.2-fold (Fig. 2). Lycopene (10 µg/ml) and β-carotene (10 and 25 µg/ml) did not show any significant reduction of the NF-κB activity (Table 3). MAPK activity in unstimulated cells (B and B/M) was already high compared with the positive control, and the carotenoid treatment did not show any further significant reduction of the MAPK activity (Table 3).

Table 3:

Effect of lycopene (L) and β-carotene (BC) exposure of Caco-2 cells on different inflammatory markers (IL-8, cyclo-oxygenase 2 (COX-2) and NO), and on Intracellular signalling cascades (NF-κB and mitogen-activated protein kinase (MAPK) activation) on Caco-2 cells, expressed per cent compared with control and absolute values (Mean values and standard deviatons)

						COX-2 activation via PGE-						NF-κB				
	Cell Viability		IL-8 production			2α production			NO production			activation		MAPK activation		
Cell exposure*	Mean	SD	Mean	SD	Mean	Mean‡	SD	Mean	Mean	SD	Mean	Mean	SD	Mean	SD	Mean
	† (%)	(%)	ǂ (%)	(%)	(pg/ml)	(%)	(%)	(pg/ml)	ǂ (%)	(%)	(μM)	§(%)	(%)	§ (%)	(%)	(pg/ml)
Blank	95.4	7.2	24.2	16.8	18.4	106.1	12.9	275.4	87.5	18.7	2.4	30.6	19.0	98.6	5.4	176.9
Blank/micelles	82.5	6.8	11.1	19.9	8.5	97.8	32.5	177.3	106.1	18.9	2.9	30.3	9.8	95.9	5.9	172.1
Blank/micelles/stimulus	100.0	11.7	100.0	20.2	76.1	100.0	36.7	199.7	100.0	16.4	2.8	100	9.5	100	12.6	179.4
L 10 μg/ml	111.2	7.3	137.6	18.9	104.7	101.6	36.7	217.5	95.9	26.1	2.6	86.5	10.0	104.8	12.7	187.9
L 25 μg/ml	111.5	7.9	138.6	11.2	105.4	103.3	32.1	237.9	102.9	18.2	2.8	-	-	-	-	/
BC 10 μg/ml	88.8	7.0	116.0	28.2	88.3	104.55	31.9	254	86.8	23.0	2.4	111.6	4.7	112.0	15.8	200.8
BC 25 μg/ml	86.2	4.3	108.0	43.9	82.2	111.96	37.5	376.2	114.5	18.4	3.2	107.3	10.9	88.6	11.1	159.0

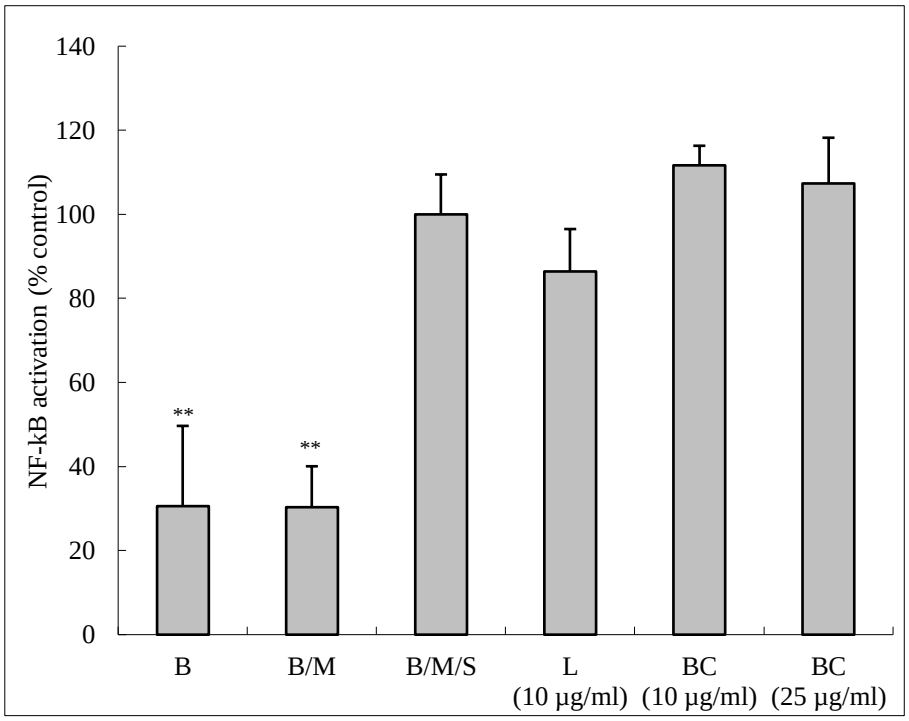
*Blank, without stimuli and micelles; blank/micelles, without stimuli but with empty micelles (negative control); blank/micelles/stimuli, with stimuli and empty micelles (positive control), *i.e*. TNF-α plus IL1-β at 100 and 25 ng/ml (28 h) and exposed to empty micelles for 24 h; L, L (10 or 25 μg/ml), cells stimulated with the TNF-α/IL1-β mixture (28 h) exposed to micelles containing either 10 or 25 μg L/ml (24 h); BC, BC (10 or 25 μg/ml), cells stimulated with the TNF-α/IL1-β mixture (28 h) exposed to micelles containing either 10 or 25 μg BC/ml (24 h).

† n 12: 3d with each six independent replicas.

ǂ n 6: total of n 6 obtained from three different experiments conducted on three different days with independent duplicates.

§n 4: total of n 4 obtained during four different days (one flask (75cm^2), harvested proteins and finally pooled for experiment.

Figure 2:



Effect of the carotenoids on the NF-κB activation of Caco-2 cells stimulated for inflammation with TNF- α (100 ng/ml) and IL-1β (25 ng/ml) for 24 h. The cells were exposed to various treatments and the NF-κB activation was measured after 28 h. Values are means from four replicates of each sample compared to the control (blank (B)/micelles (M)/stimulus (S) = 100 %), with standard deviations represented by vertical bars. B/M, B+M; B/M/S, B+M+S; L, lycopene, BC, β-carotene. Mean values were significantly different by Dunnet's test ($P < 0,01$)

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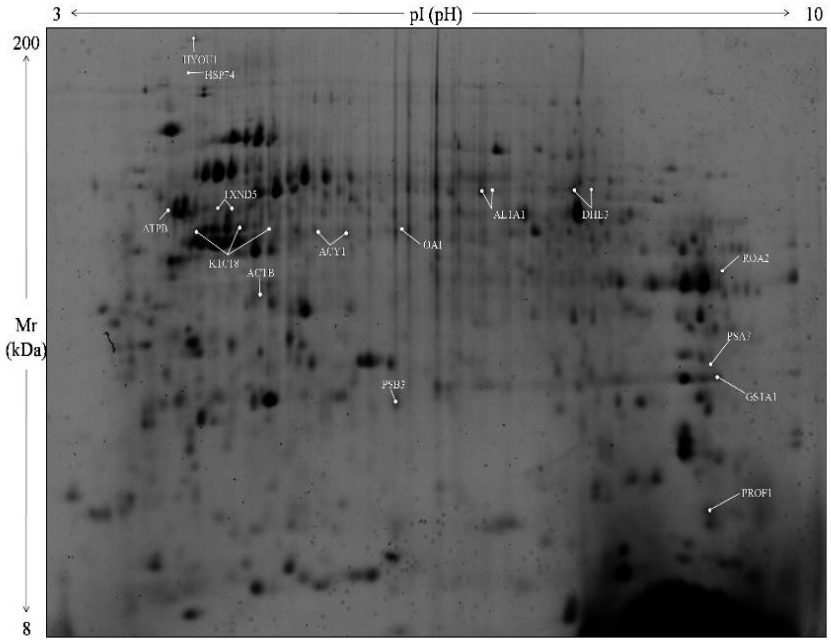
1.3.3 Proteomic analyses

The analysis of the different treatments revealed that overall and within all pairwise comparisons, sixty-five spots were differentially regulated in their expression ($P < 0.05$, expression ratio at least ± 1.3-fold) and had a unique identification, belonging to fifty-two different proteins (Fig. 3). Out of these, fifteen were differentially regulated in their expression due to the lycopene and β-carotene treatments (Table 4), compared with cells treated with empty micelles and inflammatory stimuli. The fifteen proteins were then classified with respect to their localisation and biological pathway in which they were involved, according to Gene Ontology (http://www.geneontology.org). Most of the proteins were intracellular proteins, predominantly located in the cytoplasm (67%), predominantly involved in metabolic pathways (34%) and in the stress response (40 %). Among the fifteen differentially expressed proteins, eleven were differentially expressed following the treatment with β-carotene at 10 µg/ml, three were differentially expressed by β-carotene at 25 µg/ml, one protein was differentially expressed in both b-carotenoid treatments and one protein was differentially expressed by lycopene (10 µg/ml). From the eleven differentially expressed proteins following the β-carotene (10 µg/ml) treatment, 82% were down-regulated, while the three differentially expressed proteins due to the β-carotene treatment at 25 µg/ml were all up-regulated, compared with the positive control (M+S). The only protein that was differentially expressed following the lycopene (10 µg/ml) treatment was up-regulated (Table 4).

Figure 3:



Differentially expressed protein spots identified following two-dimensional in-gel electrophoresis (2D-DIGE) analysis. The 2D-DIGE analysis was done on the whole protein extract from Caco-2 cells which were treated with empty micelles and inflammation stimulus for 24 h (TNF-α (100 ng/ml) /IL-1β (25 ng/ml)), treated with lycopene (10 μg/ml) or β-carotene (10 and 25 μg/ml) for 24 hours plus the inflammation stimulus for 28 h. Proteins were separated in the first dimension on a nonlinear pH gradient and in the second dimension on a SDS-PAGE gel. Comparisons were made for each treatment against blank + micelles + inflammation stimulus (control).

Table 4:

Up- and down-regulation of the fifteen differentially expressed proteins following carotenoid exposure compared with the inflammatory-stimulated blank (positive control, blank + micelles (M) + Stimulus (S))*.

					Blank + M + S		
Spot no.	*T* Test	Fold change†	Accession no.ǂ	Protein name	L (10 ug/ml)	BC (10 ug/ml)	BC (25 ug/ml)
104	0,021	-1,78	P34932	Heat shock 70kDa protein 4	-	Up-regulated	-
665	0,044	1,82	P00352	Retinal dehydrogenase 1	-	Down-regulated	-
695	0,036	1,68	P00367	Glutamate dehydrogenase 1 mitochondrial	-	Down-regulated	-
748	0,002	-2,09	Q8NBS9	Thioredoxin domain-containing protein 5	-	-	Up-regulated
761	0,027	-1,63	P06576	ATP synthase subunit β mitochondrial	-	-	Up-regulated
842	0,026	-1,74	P05783	Keratintype I cytoskeletal 18	-	-	Up-regulated
875	0,029	1,67	P04181	Ornithine aminotransferase mitochondrial	-	Down-regulated	-
879	0,030	1,69	O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	-	Down-regulated	-
1149	0,024	-1,64	P22626	Heterogenous nuclear ribonucleoproteins A2/B1	-	Up-regulated	-
1156	0,047	1,52	P60709	Actin cytoplasmic 1	-	Down-regulated	-
1533	0,037	1,95	O14818	Proteasome subunit α type-7	-	Down-regulated	-
1580	0,008	1,93	P08263	Glutathione S-transferase A1	-	Down-regulated	-
1671	0,028	1,48	P49720	Proteasome subunit β type-3	-	Down-regulated	-
1931	0,035	-1,68	P07737	Profilin-1	Up-regulated	-	-
2765	0,001	1,94	Q9Y4L1	Hypoxia up-regulated protein	-	Down-regulated	-

* Among the eleven differentially expressed proteins during BC (10 µg/ml) treatment, 73% were down-regulated compared with the positive control. Among the three differentially expressed proteins during the BC (25 µg/ml) treatment, all were up-regulated. For the L (10 µg/ml) treatment, only one protein was differentially expressed and was up-regulated.

†Fold change: protein expression compared to control (mean expression control/mean expression treatment).

ǂ Swissprot accession number.

1.4 Discussion

In the present study, we investigated the hypothesis that individual carotenoids might exhibit anti-inflammatory properties on cells resembling the gastrointestinal epithelium, and could constitute a preventive dietary strategy against chronic gut diseases such as UC or CD. We tested the influence of β-carotene and lycopene in artificial micelles on inflammatory-stimulated Caco-2 cell production of several inflammatory endpoints, including IL, NO, COX-2 activity, intracellular signalling pathways (NF-κB and MAPK) and proteomics. While individual carotenoids at realistic dietary concentrations did not significantly affect inflammatory markers, several proteins were differentially regulated, indicating that carotenoids could alter the general metabolism of these epithelial cells. To our knowledge, this is the first time that the impact of carotenoids on inflammatory markers of the gut epithelium has been studied. Both β-carotene and lycopene are found in a wide range of frequently consumed fruits and vegetables. Concentrations tested (10 and 25 μg/ml) represented high, but still plausible concentrations that could be reached following, for example, consumption of approximately 300 g carrots, assuming a total volume of gastrointestinal liquids of 1 litre (29). The viability studies conducted indicated that the carotenoids added did not result in adverse effects. Furthermore, micelles alone, without lycopene and β-carotene, did not result in any significant changes of the inflammatory markers studied. Both TNF-α and IL-1β used for inflammation stimulation are activators of inflammatory pathways, and have been previously used to study the impact of cytokines on inflammation (30). TNF-α and IL-1β in the present study resulted in inflammatory

stimulation, as determined by the 4.1-fold increase in IL-8 and the 3.2 -fold increase in NF-κB, albeit they did not significantly change PGE-2α, NO and MAPK. It may be that the exposure time of 24h was not long enough to induce the production of PGE-2α, NO and to activate MAPK. NO production, for example, requires more steps than the production of IL-8. IL-8 expression follows binding of NF-κB to the promoter region of the IL-8 gene (31). NF-κB is responsible for the expression of inducible NOS, which then catalyses the reaction of L-arginine into NO (32). PGE-2α synthesis involves even more enzymatic steps for activation compared with, for example, IL-8 and NO. Following binding of NF-κB, COX-2 catalyses the reaction of arachidonic acid to PGG-2 and further to PGH-2. The PG synthase E then catalyses the reaction to PGE-2α (33). Another factor might be the status of the cells. While our experiments were conducted with 1-week differentiated cells, proliferating cells could respond differently. Van de Walle *et al.* (30) showed that PGE-2 concentration was higher in proliferating cells than in differentiated cells, while NO production was detectable only in 3-week differentiated cells. However, inflammation induction following IL-8 and NF-κB was slightly lower in the present study compared with a similar earlier study (34), with a 7.5- and 5.8-fold increase, respectively. Thus, the inflammation in the present study was rather indicative of a low, chronic type. IL-8 is a chemokine responsible for the chemotaxis of neutrophiles and their degranulation (35). We did not observe any significant changes of this marker following carotenoid exposure, neither of the regulation of NF-κB, being in line with the theory that IL-8 is regulated via NF-κB (36). As with IL-8, PGE-2α is likewise a major mediator of intestinal

inflammation (37), resulting from COX-2 activity and arachidonic acid. While in a previous study (38), a similar concentration of arachidonic acid resulted in a 5.6-fold increase of PGE-2, in the present study, PGE-2α production remained low during all experiments, suggesting that carotenoids were not able to modify the activity of COX-2 significantly. NO plays a central role in human IBD (39). In the present study, no significant change in NO concentration following carotenoid exposure was observed. Contrarily, Romier-Crouzet *et al.* (38) showed an increase in NO production after inflammatory stimuli by 1.9-fold; however, stimulation was done for 48h and with a mixture of interferon-γ, IL-1β, TNF-α and lipopolysaccharide. We also investigated the effect of carotenoids on the NF-κB cascade. NF-κB has many target genes, playing an important role in inflammation. In the present study we determined inducible NOS through NO production, COX-2 through PGE-2α production and the IL-8 gene through the IL-8 production. All compounds have been reported to be impacted via the NF-κB pathway (16,32,36), albeit they may also be regulated by NF-κB-independent pathways (40). In this study, the stimulation of Caco-2 cells with TNF-α and IL-1β resulted in a significant increase in NF-κB compared with the unstimulated B, indicating activation of NF-κB-dependent pathways, as shown by the increased IL-8 production in the present study. The MAPK cascades are also involved in the production of inflammatory mediators, three are well-characterised in mammals: extracellular signal-regulated kinases (ERK1/ERK2); c-Jun N-terminal kinases (JNK1, JNK2 and JNK3); p38 kinases (41). The target genes of the p38 kinases are mostly cytokines, and thus play a

crucial role in inflammatory responses (41). As the immunoassay kit used during our study detected the total produced p38 MAPK (independently of phosphorylation state), we already observed high concentrations of p38 in unstimulated cells, but no further impact following the inflammation trials. Contrarily to earlier studies suggesting anti-inflammatory properties of β-carotene and lycopene in cellular trials as measured by NO, PGE-2, inducible NOS and COX-2 (14,42), in the present study, the two carotenoids did not affect NO, IL-8 and COX-2 activity, further confirmed by NF-κB and MAPK. The reasons remain speculative. It has been argued that dietary antioxidants, including β-carotene and lycopene, possess biphasic effects, with higher concentrations resulting even in negative, *i.e*. pro-oxidative, effects (43). Despite no effects on selected markers, proteomic analysis revealed that a total of fifteen proteins were differentially expressed following the carotenoid treatments. These proteins were predominately involved in metabolic pathways. About two-thirds of these differentially expressed proteins were present in the cytosol. Lycopene appeared to have a lower impact compared with β-carotene, as only one protein (profilin-1) was differentially expressed. One explanation could be the low cellular uptake as opposed to β-carotene (44), also observed in the present study. Profilin-1 is an actin-binding protein, involved in the regulation of the cytoskeleton assembly. It is also able to bind cell membrane components such as phosphatidyl-inositol-4,5-bisphosphate and to regulate the formation of inositol triphosphate, thus acting as a signalling protein (45). At intermediate concentrations (10 μg/ml), β-carotene resulted rather in a down-regulation of differentially

expressed proteins. In contrast, changes induced by β-carotene at 25 µg/ml were limited to only three proteins, all of which were up-regulated. These included mitochondrial ATP synthase subunit b, part of the catalytic domain of the ATP synthase and responsible for the formation of ATP from ADP and inorganic phosphate; cytoskeletal keratin type I constituting a component of the intermediate filaments exerting vital structural functions in eukaryotic cells and also involved in regulatory processes, including apoptosis modulation (46); and thioredoxin domain-containing protein 5, involved in the resistance to nitrosative stress (47). Most proteins down-regulated by β-carotene at 10µg/ml are potentially involved in inflammation. For example, glutathione transferase participates in the elimination of xenobiotics, and glutathione is a major endogenous antioxidant, important for controlling pro-inflammatory processes (48). The reduced activity of these enzymes observed may be representative of a decreased cellular stress following carotenoid exposure. Hypoxia up-regulated protein 1 has an important role in response to hypoxia-induced stress, modulating apoptosis. Furthermore, up-regulated were heterogeneous nuclear ribonucleoproteins A2/B1 and heat shock protein 70 kDa protein 4, the former being involved in mRNA maturation and processing, the latter in folding and assembly of proteins, as well as in stress response. Caco-2 cells have been frequently employed in various studies; however, it is also important to point out the limitations of this model. While Caco-2 cells resemble the human intestinal epithelium, they are of neoplastic origin and might therefore differ in features from the natural intestine. Caco-2 cells further are monolayers mimicking only enterocytes, therefore lacking, for

example, the mucus layer. Nevertheless, Caco-2 cells express various transporter and efflux proteins and have been frequently used for investigating bioavailability aspects of various compounds (49,50) and also for stimulating intestinal inflammation (30,38). In addition, Caco-2 cells express various transporter proteins and efflux proteins. Finally, it cannot be excluded that the artificial system chosen here for carotenoid emulsification could have compromised the uptake of carotenoids and therefore their influence on Caco-2 cells, as artificial micelles could differ from micelles formed during the normal gastrointestinal digestion, for example in terms of size and release kinetics of carotenoids. In summary, β-carotene and lycopene were not able to show significant effects on selected inflammatory mediators in stimulated Caco-2 cells, albeit the proteomic results indicate that several pathways related to inflammation, such as antioxidative enzymes, were involved. More studies in this domain, examining different carotenoids, and perhaps mixtures representing more natural conditions, are warranted.

1.5 Acknowledgements

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2. Introduction (Chapter 2):

The introduction chapter (chapter 2 & 3) corresponds to two review articles: Anouk Kaulmann and Torsten Bohn (2016), “Bioactivity of Polyphenols: Preventive and Adjuvant Strategies toward Reducing Inflammatory Bowel Diseases—Promises, Perspectives, and Pitfalls,” Oxidative Medicine and Cellular Longevity, vol. 2016, Article ID 9346470, 29 pages and Kaulmann, A.; Bohn,T. (2014) “Carotenoids, inflammation, and oxidative stress – implications of cellular signaling pathways and relation to chronic disease prevention”, Nutrition Research, Volume 34, Issue 11, 907-929.

Bioactivity of Polyphenols – Preventive and Adjuvant Strategies toward Reducing Inflammatory Bowel Diseases – Promises, Perspectives, and Pitfalls

Abstract

Inflammatory bowel diseases (IBDs) are characterized by autoimmune and inflammation-related complications of the large intestine (ulcerative colitis) and additional parts of the digestive tract (Crohn's disease). Complications include pain, diarrhoea, chronic inflammation, and cancer. IBD prevalence has increased during the past decades, especially in Westernized countries, being as high as 1%. As prognosis is poor and medication often ineffective or causing side effects, additional preventive/adjuvant strategies are sought. A possible approach is via diets rich in protective constituents. Polyphenols, the most abundant phytochemicals, have been associated with anti-inflammatory, antioxidant, immunomodulatory, and apoptotic properties. Locally reducing oxidative stress, they can further act on cellular targets, altering gene expression related to inflammation, including NF-κB, Nrf-2, Jak/STAT, and MAPKs, suppressing downstream cytokine formation (e.g.,IL-8,IL-1β, and TNF-α),and boosting the bodies' own antioxidant status (HO-1, SOD, and GPx). Moreover, they may promote, as prebiotics, healthy microbiota (e.g., Bifidobacteria, *Akkermansia*), short-chain fatty acid formation, and reduced gut permeability/improved tight junction stability. However, potential adverse effects such as acting as prooxidants, or perturbations of efflux transporters and phase I/II metabolizing enzymes, with increased uptake of undesired xenobiotics, should also be considered. In this review, we summarize current knowledge around preventive and arbitrary actions of polyphenols targeting IBD.

2.1. INTRODUCTION – PREVENTIVE STRATEGIES FOR IBD

2.1.1 General aspects

Inflammatory bowel diseases (IBDs) are on the rise. With annual incidence rate (newly diagnosed diseases) up to 1 ‰ and a prevalence of 1% in many developed countries [1], this complication is affecting considerably more people than in the past, for reasons unknown. Crohn's disease (CD) and ulcerative colitis (UC) are the main forms of the disease, with CD resulting in manifestations in the small and large intestine, while UC is confined to the colon. Typically, the disease manifests itself before 30 years of age, and most likely genetic predisposition followed by autoimmune reactions does play a role in their aetiology, though concrete reasons or triggers are not understood. Symptoms include diarrhoea, abdominal pain, cramping, fever, weight loss, wasting, internal bleeding, and ultimately cancer. In both diseases, the epithelial lining of the gut is in part destroyed, resulting in perturbed permeability of the mucosal barrier, malabsorption of nutrients, and absorption of compounds by-passing the enterocytes, causing, for example, allergic reactions, a circumstance often described as "leaky gut syndrome"[2,3]. Many subjects present with low concentrations of essential micronutrients such as vitamins and minerals, especially zinc, iron, selenium, vitamin B12, and vitamin D [4–7], possibly (a) due to low dietary intake and avoidance of many food products expected to cause digestional discomfort, (b) due to diarrhoea, loss of blood, and malabsorption in the inflamed areas, and

perhaps (c) due to enhanced metabolism/turnover of some of these essential micronutrients (such as antioxidant vitamins).

2.1.2 Pathophysiological description of condition

Several differences between CD and UC exist. First, while CD can affect both the small and the large intestine (in addition to mouth and stomach), UC is limited to the colon. However, most typically, CD affects the lower parts of the small intestine (distant ileum) and the upper parts of the colon. Second, another distinction is that while CD may affect the entire gut wall, UC typically affects only the inner lining (mucosa, submucosa) of the epithelium. Third, CD may affect certain areas and leave intermittent parts ("skip areas") intact, which is not observed in UC [8]. In CD, crypt inflammation and abscesses may turn into mucosal oedema, thickening of the bowel wall, and fibrosis and fistula development (extending to other organs such as the bladder), among others. UC typically starts with the rectum, mucosal ulcers are common, and fistulas and abscesses are absent.

Why the epithelial lining and additional adjacent tissues are inflicted is not entirely understood, but autoimmune reactions appear to be involved [9], characterized by local spots of increased inflammation, including infiltration of immune cells. Several cell types are involved in this response, including absorptive enterocytes, mucus-producing goblet cells, enteroendocrine cells (secreting hormones such as cholecystokinin), paneth cells (required for bacteria defence), microfold cells (M-cells, taking up antigens via endocytosis), and additional infiltrating cells of the immune system, such as neutrophils. It has been reported that secondary lymphoid

tissues, for example, Peyer's patches, and tertiary lymphoid tissues can respond to antigen stimuli, releasing cytokines and antibodies (IgA, [10]). Cell surface receptors (toll-like receptors (TLRs) and nod-like receptors (NLRs)), located on many cells of the immune system, infiltrating to the diseased tissue, may sense pathogen associated molecules. Here in lies an important interaction with the gut microbiota, as certain bacteria such as Bacteroides can interact with, for example, T regulatory cells and macrophages, stimulating anti-inflammatory IL-10 production, while other bacteria may induce T-helper-(Th-)17 cells, fostering inflammation.

However, this process is characterized not only by local inflammation (Figure 1), but by a systemic low chronic inflammation, with increased concentrations of circulating cytokines, especially IL-8, TNF-α, and IL-1 β [11], and other general markers of inflammation such as C-reactive proteins (CRP) [12]. It is believed that IBD is mostly triggered and aggravated by TNF-α released from infiltrating immune cells (macrophages), followed by increased concentrations of the cytokines IL-6 and IL-1β [13] and possibly IL-12 (especially for CD) and IL-13 (especially UC) [14], and reduced concentration of the anti-inflammatory cytokines IL-10 and IL-4 [15]. Immune cells (neutrophils and macrophages) also produce a number of reactive oxygen species (ROS) in order to trigger further inflammation, resulting in reduced plasma antioxidant activity. This typically goes along with increased levels of myeloperoxidase (MPO, producing ROS from hydrogen peroxide [16]), causing enhanced formation of lipid oxidized products (LOP) such as malondialdehyde (MDA) and advanced oxidized protein products (AOPP, Figure 2). This in turn

may be accompanied with increased levels of markers of (nonenzymatic) oxidative stress, such as F2-isoprostanes [17].

Despite the fact that the precise reasons for the increased prevalence of IBD are still controversially discussed, certain environmental aspects appear to play a role (Table 1), such as smoking, hygiene, certain microorganisms, use of oral contraceptives (OCPs), nonsteroidal anti-inflammatories (NSAIDs), antibiotics, appendectomy, breastfeeding, ambient air pollution [18], the gut microbiota [19], and certain diet related habits, such as high fat consumption, consumption of refined sugars, and low vitamin D intake, at least according to some studies[18]. A genetic predisposition has also been reported [20, 21]. Certain mutations have been revealed, such as (for CD subjects) the gene encoding for NOD2 (nucleotide-binding oligomerization domain 2) [14]. A similarity between UC and CD with the aetiology of celiac disease (CeD) also exists, with the main difference that people may stay asymptomatic with CeD, as long as the known antigen triggering the disease, that is, gluten, is avoided [22].

Fig. 1. Factors involved in the origin and progression of IBD, via inflammation and oxidative stress. For abbreviations see footnote table 3.

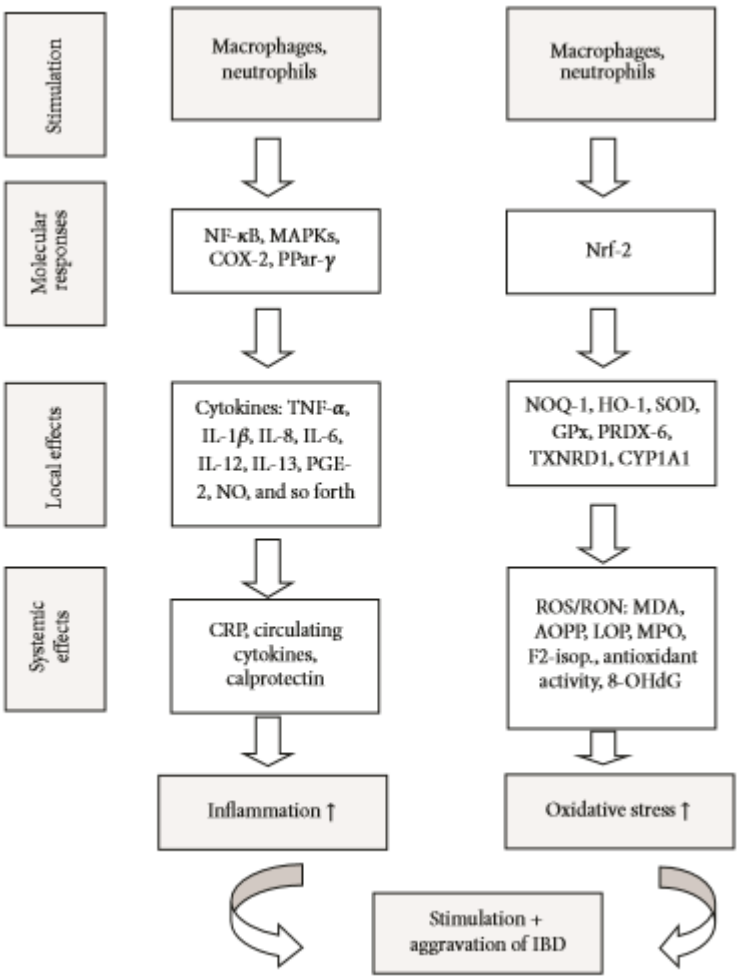

Fig. 2. Summary of mechanisms via which polyphenols may positively or negatively influence the development of IBD.

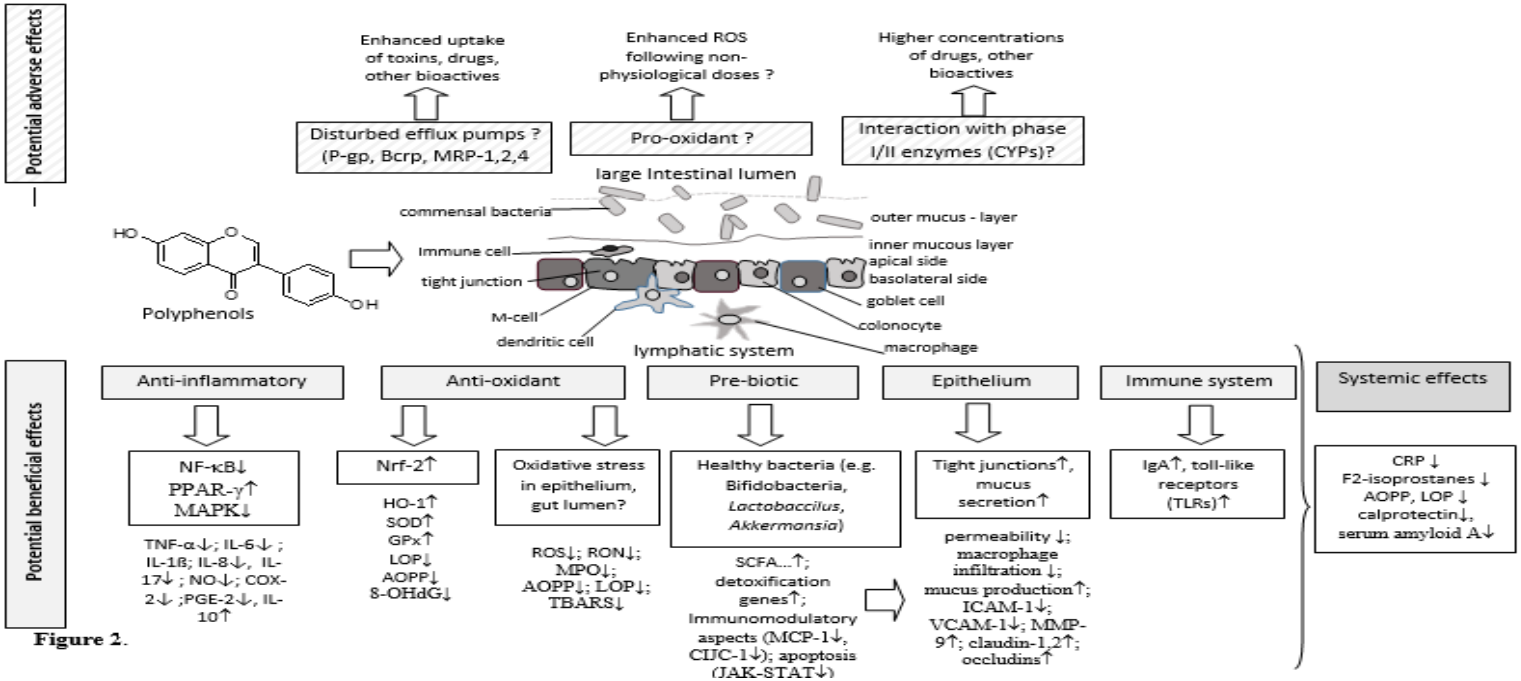

Figure 2.

For abbreviations see footnote table 3.

Table 1: Overview of major risk-factors and suggested mechanisms involved in the development of IBD.

Table 1: Overview of major risk factors and suggested mechanisms involved in the development of IBD.

Risk factor	Influence: positive (+), negative (−)	Mechanism	Reference
Genetic predisposition	+/−	Genes involved in inflammation and oxidative stress responses and in immune function (histocompatibility complex)	[19, 33]
Smoking	−	Altered blood flow, enhanced cytokine formation, immunomodulatory, influencing mucus production	[18]
Air pollution (NOx, SO_2 …)	−	Unclear: proinflammatory response to air particles?	[18]
Enhanced hygiene	−	Unclear: reduced exposure early in life to microorganisms. Reduced IBD prevalence found for growing up on farms, living in crowded homes, consuming unpasteurized milk	[18]
Microbiota	+/−	Immunomodulatory properties, production of anti-inflammatory compounds. Some bacteria strains associated with negative effects (e.g., Clostridia), others with positive effects, such as Bifidobacteria, possibly due to enhanced gut barrier properties, production of SCFA	[34]
Diet, probiotics	+	Immunomodulatory properties, production of anti-inflammatory compounds, see microbiota	[28]
Diet, prebiotics (fiber)	+	Favouring healthy microbiota (Bifidobacteria…), production of anti-inflammatory SCFA, lowering of pH	[28]
Diet, vitamin D	+	Immunomodulatory, protection of barrier	[35]
Diet, dietary fiber	+	Production of anti-inflammatory SCFA, increasing fecal bulk and lowering concentration of compounds with adverse effects	[17, 26]
Diet, vitamins C, E	+/−	Unclear: antioxidants may reduce ROS parameters, normalize abnormally low tissue levels in subjects with IBD	[36]
Diet, PUFAs (ω-3)	+	Anti-inflammatory effects	[37]
Diet, polyphenols	+	Antioxidant effects (reducing ROS), anti-inflammatory properties, altering genetic expression via NF-κB, Nrf-2, improving barrier properties, immunomodulatory	[13, 38–40]

PUFAs: polyunsaturated fatty acids, ROS: reactive oxygen species, and SCFA: short-chain fatty acids.

2.1.3 Strategies for ameliorating IBD.

Strategies to resolve complications have been including the administration of nonsteroidal anti-inflammatory drugs [23], steroid anti inflammatories, typically targeting TNF-α reduction [24] or, for the worst cases, surgical removal of the inflammatory afflicted areas. A standard therapy is the administration of 5-aminosalicylic acid (a nonsteroidal anti-inflammatory drug), which reduces (via cyclooxygenase-2 (Cox-2)) the formation of leukotrienes and prostaglandins, focussing on blocking inflammatory processes, that is, their mediators [8]. Nevertheless, drugs usually do not result in complete cure, and relapse rates, even after operation, remain relatively high.

As a consequence, preventive strategies appear as a prudent approach to avoid IBD, which is complicated by the fact that the causes of IBD are not fully understood. Nevertheless, a diet rich in fruits and vegetables has been shown to be able to reduce the incidence/prevalence of IBD [25], perhaps due to positive effects of dietary fiber [26]. Dietary fiber promotes the production of short chain fatty acids (SCFAs) in the colon, and these have been reported to possess anti-inflammatory and immunomodulatory effects. Fibre would also reduce colonic pH, inhibiting the growth of potential pathogenic microbes. This has been suggested especially for selected dietary fibers such as prebiotics [27]. Consequently, also probiotics have been promoted to reduce IBD symptoms; for example, some positive effects were seen for probiotics and UC [28], maintaining remission, while effects on CD have not been shown so far.

An additional class of compounds, often affiliated in the fruit and vegetable matrix with dietary fiber, which has recently attracted much attention, are polyphenols, the most abundant secondary plant compounds or phytochemicals. Polyphenols, sometimes also termed phenolics, constitute a broad class of compounds, comprising over 7000 compounds. They can further be subdivided into flavonoids (such as isoflavonoids and anthocyanins) and non flavonoids (e.g., stilbenes, phenolic acids, coumarins, and tannins). Their concentration in some fruits and vegetables (Table 2) can be as high as several 100 mg/100g [29], and their per capita intake typically ranges around 1 g/d [30]. It is also important to consider that the majority of polyphenols (possibly as high as 90–95 %) is not absorbed but reaches the colon [31] and is thus available as a substrate for fermentation. A review on the most prominent polyphenol containing food items was published by Pérez-Jiménez *et al.* [32]. In the following, we try to summarize the various pathways via which polyphenols could act on the development of IBD. More specifically, aspects related to

(a) direct and indirect antioxidant,

(b) anti-inflammatory,

(c) gut microflora,

(d) other properties with respect to epithelium protection, such as their influence on tight junctions,

are discussed, as these appear to constitute major mechanisms of action of polyphenols with respect to potential health benefits targeting IBD prevention or amelioration. In addition, potential negative effects of polyphenols are also briefly discussed. For this

purpose, searches in PubMed for all years of English literature were carried out, employing the search terms “(polyphenol? or phenolic? or flavonoid) and (IBD or inflammatory bowel disease or Crohn’s disease or ulcerative colitis)”.

Table 2. Selected food items rich in various polyphenol classes, including potential health beneficial effects

Food item	Edible part	Concentration (mg/100g)*	Major polyphenol (classes)	Reference regarding content	Suggested health effects, selection[$]	Reference regarding health effect
Apples	Peel Flesh Total	-120[$] 0.2-0.9 ca. 5-50	Phlorizin, phenolic acids: chlorogenic acid, quercetin	[41]	Blood glucose lowering, anti-inflammatory, lowering colonic inflammation	[42-44]
Blackberries	Whole	130-405	Anthocyanins, flavanols: EC, phenolic acid: ellagic acid	Phenol explorer[1]	Anti-inflammatory, anti-IBD	[1, 2]
Blueberries, highbush	Whole	160-480	Anthocyanins, flavonols:quercetin, phenolic acids : chlorogenic acid	Phenol explorer	Anti-inflammatory, anti-IBD	[3]
Cacao	Bean, powder	300-1100	Flavanols: EC,	Phenol explorer	Anti-inflammatory, against heart failure	[4, 5]
Chestnut, raw	Whole nut	547-1960	Hydroxybenzoic acids: gallic acid, ellagic acid, tannins	Phenol explorer	Anti-inflammatory	[6]
Chocolate	Dark	150-425	Flavanols: epicatechin, hydroxycinnamic acid: ferulic acid	Phenol explorer	Reducing CVD, anti-inflammatory	[7]
Cloves	Seasoning, dried	1200-17500	Hydroxyhenylpropenes: eugenol, acetyl eugenol	Phenol explorer	Anti-inflammatory, Anti-ulcer	[8]
Coffee	Beverage, filtered	90	Phenolic acids: chlorogenic acid	Phenol explorer	Improved blood lipids, improved glucose handling, anti-inflammatory, but increases IBD symptoms	[9, 10]
Curcuma	Spice, whole	200[+]	Curcuminoids, flavonoids, phenolic acids	[11]	Anti-IBD, anticancerogenic, anti-inflammatory	[12]
Grapefruit	Flesh	15-115	Flavonoids, phenolic acids	Phenol explorer	Anti-inflammatory	[13]
Green tea	Drinkable extract	29-103	Flavanols: EC, EGCG,	Phenol explorer	Anti-inflammatory, anticolitic	[14]
Olive oil, extra virgin	Whole oil	4-200	Tyrosols, lignans: pinoresinol, phenolic acids, hydrolysable tannins	Phenol explorer	Anti-inflammatory, reducing CVD	[15]
Paprika, green	Whole fruit	0.3-10	Flavonoids: luteolin glucosides, hydroxycinnamic acids	Phenol explorer	Anti-inflammatory	[16]
Peppermint	Seasoning, dried	450-26000	Flavonoids: eriocitrin, hydroxycinnamic acids: rosmarinic acid	Phenol explorer	Anti-inflammatory	[17]

Table 2: Continued

Food item	Edible part	Concentration (mg/100g)*	Major polyphenol (classes)	Reference regarding content	Suggested health effects, selection[$]	Reference regarding health effect
Pomegranate	Juice	240	Punicalagin (an ellagitannin)	[18]	Anti-inflammatory, anti-IBD	[19]
Potato	Peel Flesh Total	180-5000[#] 1-1000[#] 10-50	Phenolic acids: chlorogenic acid,	[20] Phenol explorer	Glycoalkaloids may increase IBD, anthocyanins anti-inflammatory effects in colored potatoes	[21, 22]
Plum, dark	Total	130-240	Phenolic acids: chlorogenic acid, procyanidins, anthocyanins	Phenol explorer	Anti-inflammatory, anti-oxidant,	[23, 24]
Red wine	Final product	25-300*	Phenolic acids, anthocyanins, tannins, stilbenes (resveratrol)	Phenol explorer	Improved blood lipids, anti-inflammatory, anti-IBD	[25, 26]
Soy	Flour	140-900	Isoflavonoids: daidzein, glycitein, genistein and glucosides	Phenol explorer	Improved blood lipids, anti-apoptotic effects, anti-inflammatory, anti-IBD	[27, 28]
Spinach	Leaf	30-290	Flavonols	Phenol explorer	Anti-inflammatory, anti-IBD	[29]
Wheat	Whole grain	85-220	Phenolic acids: hydroxybenzoic acids, hydroxycinnamic acids	Phenol explorer	Unclear, enhancing celiac disease, controversial effects	[30]

*In juices and wine: mg/100 mL; [1]www.phenol-explorer.eu; [#]Calculated from dry weight assuming 80% water content. Note that content in purple potatoes is ca. 5 times higher in polyphenol content than other varieties; [$] concentration in mg/cm^2, CVD= cardiovascular diseases, EC=epicatechin, EGCG=epigallocatechin, [+]total polyphenols with Folin-Ciocalteu, [$]effect refers to observation with whole food or respective extracts, but is attributed – at least in part - to the respective polyphenols.

2.2 Insights from human trials

2.2.1 Epidemiological insights

A limited number of epidemiological trials have suggested a positive association between fruit and vegetable consumption and IBD. For example, in a prospective cohort study with over 170.000 women participating in the Nurses' Health Study [115], subjects consuming the most dietary fiber had a 40% lower risk of developing CD (OR 0.59, 95 CI 0.39–0.90). Positive influences on the gut microbiota and the aryl-hydrocarbon receptor (AhR), mediating protection against xenobiotics, were discussed. Interestingly, fiber from fruits showed greatest effects, while fiber from cereals and whole grains appeared not to alter the risk, which may have been due to additional effects of polyphenols, as extractable polyphenols are especially associated with various fruits. It should also be noted that fiber intake did not appear to influence UC in this study. Similarly, a systematic review of the literature suggested that the intake of fiber and high fruit intake was associated with a decreased risk of CD and high vegetable consumption with a decreased risk of UC [116]. An additional epidemiological finding is that newly diagnosed paediatric patients with CD were reported to have a lower fruit and vegetable intake compared to healthy subjects [117], though it is not clear whether this constitutes a cause or rather a consequence of IBD.

2.2.2 Intervention trials with IBD patients

Unfortunately, there are only a very limited number of human trials available that have focussed directly on IBD with respect to

polyphenol intervention (Table 3). Studies in general have incorporated only few subjects, as low as 10 per group, lasting from 4 weeks to 2 years, and included the administration of curcumins, red wine, blueberries, apples, cacao, and pycnogenol, up to approx. 2 g/d. A human study by Chiba *et al.* [76] with 22 CD subjects showed that a semi vegetarian diet, richer in plant foods, and therefore polyphenols, was more successful in maintaining remission over 2 years, compared to an omnivorous diet (94 versus 33 %). An earlier trial was conducted with curcumin, a rather apolar polyphenol of limited bioavailability. In their randomized, double blind, placebo controlled multicentre intervention trial, Hanai *et al.* [75] administered 2 g of curcumins plus medication per day over 6 months to 89 UC patients. A significant improvement in recurrence rate and morbidity parameters associated with UC (clinical activity index and endoscopic index) was found. It cannot be excluded that curcumin, in addition to direct effects, also enhanced the bioavailability of the prescribed medication, due to interactions either at various efflux pumps and/or via altered phaseI/phaseII metabolism [30].

Koláček *et al.* [78] investigated the effect of administering pycnogenol, a polyphenolic extract from the maritime pine (*Pinus pinaster*) bark, containing 70 % procyanidins, at 2 mg/kg body weight, over 10 weeks to 15 CD patients in remission, and compared the effects to 15 healthy controls. However, controls were not treated, not allowing for an accurate comparison between the groups. Compared to healthy controls, CD patients showed higher levels of Cu/Zn superoxide dismutase (SOD) and increased oxidative damage of proteins. Markers of inflammation such as calprotectin (a protein

produced by neutrophils and associated with systemic inflammation) and CRP were negatively associated with total plasma antioxidant activity (TAC). Following intervention, most parameters, including F2-isoprostanes, CRP, and reduced glutathione (GSH), remained rather unchanged when comparing before and after intervention, while lipoperoxide levels and AOPP were significantly reduced, and SOD significantly increased following intervention. Thus, while markers of inflammation remained generally unchanged, markers of oxidative stress were significantly reduced, making this the first study to directly investigate the effects of hydrophilic polyphenols in IBD patients.

Short-term interventions have also been conducted, though with rather more questionable results, in part as inflammation processes are less likely to be altered drastically during short-term trials. However, children with gastroenterological discomfort receiving a novel polyphenol based prebiotic (2 ounces of Preliva (Goodgut INC, USA), rich in Japanese honeysuckle, grape, and pomegranate, among others) within a single dose in a placebo controlled trial experienced significantly less stomach pain and discomfort compared to the placebo group, though no bacteria cultures were measured and no dosing was reported [118]. More such studies, with preferably mid-long-term administration of polyphenols, are much desired.

2.2.3 Studies with healthy (non-IBD) subjects

Other studies have accumulated somewhat more indirect benefits of polyphenols with respect to IBD. In a trial by Clemente Postigo *et al.* [79], the effect of red wine (RW), dealcoholized red wine (DRW), and gin consumption on 10 healthy adults was investigated in a randomized cross-over trial over 20 days (272 mL/d wine or 100 mL/d for the gin). Endpoints investigated included serum endotoxin and LPS (lipopolysaccharide-) binding protein (LBP), in addition to fecal microbiota. No significant differences were detected with regard to serum endotoxin and LBP changes with gin or DRW. However, following RW consumption, numbers of Bifidobacteria and *Prevotella* significantly increased and correlated negatively to LPS levels, emphasizing that soluble phenolic constituents in their natural (*i.e.*, alcoholic) matrix, may improve gut flora in terms of the number of healthy bacteria. However, similar results were obtained in earlier trials, were a (nonalcoholic) cacao-flavonol drink increased Bifidobacteria and Lactobacilli numbers in the gut, reducing CRP (and TG) [77] in the serum, likewise emphasizing potential prebiotic effects of polyphenols, as higher Bifidobacteria numbers have been associated with increased gut barrier properties [119], possibly via their production of SCFA and effects on the gut barrier, reducing, for example, LPS formation [120]. That the effect in the cacao-beverage study was truly attributable to polyphenols is very likely, as a cocoa drink rich in flavan-3-ol was contrasted to a similar cacao drink low in flavon-3-ol. Another study on obese subjects demonstrated that polyphenols from red wine were able to induce likewise Bifidobacteria and Lactobacilli growth, as well as butyrate producing

bacteria [83], reducing LPS producers. However, polyphenols from red wine have also been suggested to hamper inflammatory cytokines in the gut, as found in a subset of healthy volunteers with high cytokine levels (6 out of 34), consuming red wine over 4 weeks (containing ca. 1.76 g/L polyphenols), though the exact amount consumed was not registered. Similarly, in a study with normal healthy (non-IBD) subjects consuming a blueberry beverage rich in polyphenols (375 mg anthocyanins and 128 mg chlorogenic acid per d) for 6 weeks, certain Bifidobacteria counts were more pronounced following the intervention, compared to a placebo drink [80].

Also the consumption of coffee, being rich in polyphenols (in addition to fermentable fibre), has been suggested to enhance the number of health beneficial bacteria. For example, in a study that included administering instant coffee for 3 weeks (3 cups/d) to healthy subjects, the number of Bifidobacteria significantly increased compared to the onset of the intervention [121]. Similar results were obtained for green tea intake [122], enhancing intendency the proportion of Bifidobacteria, though results for black tea were less clear and did not influence the proportion of Bifidobacteria but rather decreased overall bacteria population [123], showing also high variability of the observed results.

A few reviews have meanwhile also aimed at emphasizing the potential that polyphenols may play regarding the prevention (or as an adjuvant therapy) in IBD [13, 38–40, 124] and even other ailments of the digestive tract, starting with periodontal (gum) applications [125]. In summary however, far too little data exists regarding human trials employing polyphenols in longer intervention studies, also with

respect to the kind of polyphenols, dosing, and matrix, to clearly prove that these compounds may prevent or significantly ameliorate the progression of disease, though first trials appear promising.

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Table 3. Human intervention trials suggesting health benefits of polyphenol-intake with respect to IBD – an overview.

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of curcumin on CD patients in a RCT study	89 patients with UC, 45 given 2 g curcumin/d for 6 months	Curcumin improved both clinical activity index & endoscopic index	Curcumin suppressed morbidity associated with UC	31
Effect of different dietary interventions, prospective trial	22 CD subjects receiving semi-vegetarian vs. omnivorous diet for 2 years	Semivegetarian diet more successful in maintaining remission over 2 years vs. omnivorous diet (94 vs. 33%)	Effects of fiber and polyphenols ?	32
Effect of cacao drink on gut bacteria	22 healthy adults receiving 494 mg or (n=23) cocoa flavanols/d for 4 weeks	Significant reductions in plasma TG & CRP in group receiving high flavonol drink	Flavonol rich drink sign. increased Bifidobacteria and *Lactobacilli* populations, sign. decreasing Clostridia counts	33
Effect of pycnogenol in subjects with CD	15 children with CD receiving 2 mg/kg bw. for 12 weeks. 15 control children with no intervention	Compared to baseline, improved lipoperoxides, improved SOD, reduced AOPP	Oxidative stress related markers improved following PP consumption	34
Effect of red wine consumption on plasma LPS and gut bacteria	10 volunteers, 20 d, 272 ml of red wine (RW) with/without alcohol (DRW), or 100 ml gin	No significant differences in the change in LPS or LBP conc. between chronic RW, DRW, and gin consumption	*Bifidobacterium* and *Prevotella* amounts were significantly increased by RW and correlated negatively with LPS concentrations	35
Effect of blueberry beverage (bb) on healthy subjects	20 subjects, either consuming bb (375 mg ACNs and 128 mg CA) for 6 weeks vs. placebo	Bb enhanced Bifidobacteria counts compared to placebo	Pre-biotic effect of polyphenols	36
Effect of apple products on inflammation and gut microbiota	5 × 4 weeks crossover, whole apples (550 g/d), apple pomace (22 g/d), clear & cloudy apple juices (500 ml/d), none, n= 23 healthy adults, 75-240 mg PP/d	No effect on HDL-cholesterol, TAG, weight, waist-to-hip ratio, blood pressure, CRP, gut microbiota, insulin, IGF1, lower serum LDL for whole apples and pomace	Too high variation of bacterial changes such as Bifidobacteria, already health subjects, too short intervention period ?	37
Effect of red wine on fecal markers of inflammation	34 healthy subjects drinking RW (1.76 g/L PP) for 4 weeks	In a subgroup of 6 subjects, TNF-α, IL-6, and IFN-γ in feces was sign. reduced	Reduced inflammation via NF-κB ?	38
Effect of red-wine PP on gut bacteria of obese subjects	10 obese and 10 normal subjects receiving 272 ml RW over 30 d	PP sign. increased fecal Bifidobacteria & *Lactobacillus* & butyrate-producers (*Faecalibacterium prausnitzii* & *Roseburia*) at expense of undesired bacteria, e.g. LPS producers (*E. coli* & *Enterobacter cloacae*)	Intestinal barrier protection and SCFA production	39

ACNs: anthocyanins; AMPK: adeonsime monophosphate kinase; AOPP: advanced oxidation protein endproducts; bcrp: breast cancer cell resistance protein; bw: body-weight; CA: chlorogenic acid; CAT: catalase; CYP1A1: cytochrome P450, family 1, member 1A1; CD: Crohn's disease; COX-2: cyclo-oxygenase 2; CRP: c-reactive protein; CXCL1: chemokine (C-X-C motif) ligand, neutrophil activating; DAI: disease activity index; DSS: dextran-sodium sulphate; DRW: dealcoholized wine; EA: ellagic acid; EP: *Oenothera paradoxa;* EGCG: epigallocatechin gallate; EP: evening primrose pomace; GAE: gallic acid equivalents; GM-CSF: granulocyte macrophage colony stimulating factor; GR: glutathione-reductase; Gred: reduced glutathione; GrTP: green-tea extract; GPx: glutathione peroxidase; GSH: glutathione (reduced); GSP: grape-seed polyphenols; GSTT2: glutathione-S-transferase theta 2; ICAM: Intercellular adhesion molecule 1; IFN: interferon; IL: interleukin; iNOS: inducible nitric oxide synthase; LBP: LPS binding protein; LPS: lipopolysaccharides; Mcp-1: monocyte chemoattractant protein-1; Mdr1a (-/-): multidrug resistance targeted mutation; MDA: malondialdehyde; MPO: myeloperoxidase; MIP2: macrophage inflammatory protein 2; MRP: multidrug resistance protein; NF-κB: nuclear factor kappa B; Nrf-2: Nuclear factor (erythroid-derived 2)-like 2; NQO1: NAD(P)H dehydrogenase [quinone-1] 1. PCAM-1: Platelet endothelial cell adhesion molecule. PGE2: prostaglandin-E2; P-gp: P-glycoprotein; PP: polyphenols, PRDX-6: peroxiredoxin-6, PPAR: Peroxisome proliferator-activated receptor; RCT: randomized control trial; RW: red wine; SAA: serum amyloid α; SAPK: stress activated protein kinase; SCFA: short chain fatty acids; SOD: superoxide dismutase; TBNS: 2,4,6-trinitrobenzenesulfonic acid; TBARS: Thiobarbituric acid reactive substances; TG: triglycerides; TNF-α: tumor necrosis factor alpha; TXNRD-1: thioredoxin reductase-;. UGT1A1: UDP-glucuronosyltransferase family 1 member A1; VCAM-1: vascular cell adhesion protein 1; WB: Western blot.

2. 3. Animal studies and *in vitro* trials

2.3.1 Choice of model

With respect to animal models, typically mice or rats have been employed due to cost and handling reasons, with colitis being induced by administration of proinflammatory chemicals, mostly dextran sodium sulphate (DSS) or 2,4,6-trinitrobenzenesulfonic acid (TBNA). While animal models can mimic relatively well the inflammation (and oxidative stress) in relation to cytokine activation via molecular targets, the major disparity possibly rests in the microflora, often being different from humans [126]. However, since only about 35% of bacterial genes have been reported to be shared even between human individuals, simulating representatively the microflora is anyhow a difficult task [14]. In addition, chemical induction may not entirely reflect IBDs and all the immunological aspects involved. This may be overcome by the more recent development of genetically modified rodents, which spontaneously develop IBD [127], but those have so far found little application, due to still limited availability.

Regarding *in vitro* methods, which allow the testing of many dietary factors with in a rather short period of time (due to risk of bacterial growth and the need to refresh media), most studies have been conducted with intestinal cancer epithelial cells, especially monolayer-forming Caco-2 cells and HT-29 cells, mostly without previous simulation of gastrointestinal digestion, with occasional exceptions [67]. Disregarding digestion processes will bear the risk that changes in the polyphenol profile (see Section 3.2), as well as

matrix release, and thus bioaccessibility, are not or only poorly resembling the *in vivo* situation. However, in these models, inflammation must usually be triggered (due to otherwise very low secretion of e.g., cytokines), for which certain stimulants are added, often TNF-α,IL-1β, or LPS or a mixture there of, even though the activation via LPS has been questioned, due to lack of certain LPS receptors in Caco-2 cells (e.g., TRL4), according to some reports[128].

Thus, the major limitations of these cellular methods are that they (a) typically lack the capability of including the interaction with the colonic microflora, as these are often incompatible with the epithelial cells involved, (b) do not normally include immune cells such as macrophages, which usually do infiltrate inflamed tissues, often aggravating inflammation, (c) only allow rather short-term exposure due to the risk of additional microbiological contamination of the cell models, and (d) do not take into account changes of the polyphenol profile during preceding digestion. However, some more sophisticated models have meanwhile been developed, such as triple-cell culture models encompassing also macrophage-like cells [67], which have been coupled to preceding simulated *in vitro* digestion, and also models that allow studying the interaction with bacteria and gut cells, such as the microfluidic HUMIX model [129], which however requires complete solubilisation of compounds and works only with small volumes(<100 μL).

2.3.2 Aspects of digestion and further metabolism

As many polyphenols are considerably altered in their structure during digestion, due to either degradation or further active metabolism involving host or microbiota enzymes, it is worth mentioning at least briefly major pathways and changes for predominant polyphenols that are consumed. Equally, an understanding of these processes is important as many of the metabolites may have altered bioavailability and/or bioactivity. However, in many *in vitro* models, such changes are not accounted for, neglecting potential influences of the digestion and/or the microflora on the polyphenol profile, presenting a potential limitation for many investigations.

Following ingestion of the polyphenols, these may or may not be released from the matrix. Possibly, release of the "nonextractable polyphenol fraction," that is, encompassing especially polyphenols covalently bound to the food matrix, cannot be achieved during gastric and small intestinal digestion but may in part occur in the large intestine [130] following further fermentation of the food matrix. Additional food matrix factors influencing polyphenol bioavailability have been reviewed elsewhere [30]. As many polyphenols are present in the food matrix as glycosides (e.g., flavonoids), these are believed to require (prior to their potential absorption) cleavage by human lactase-phlorizin hydrolase, situated at the brush-border of the intestinal epithelium [131], releasing the free aglycones. Alternatively, cleavage may occur intracellularly by cytosolic beta-glucosidase [132]. In addition, low pH of the stomach may likewise cleave a fraction of the glucosides. Polyphenolesters, such as hydroxycinnamates and diferulates, have also been hypothesized to be

cleaved by human enzymes, such as carboxylesterase, present on the brush-border or intracellularly within the enterocytes [133].

Regarding bacterial fermentation, which preliminary takes place in the colon, it has been reported that the microbiome is able to result in a multitude of transformations [134], depending on the number and type of bacteria species present, the foodmatrix, and the type of polyphenols. Mostly, ring fission of, for example, flavonoids [135], demethylation, dehydroxylation, decarboxylation, and deglycosylation and reduction reactions have been reported and have been reviewed previously [136, 137]. Typical end-products may include phenolic acids, or other hydroxylated aromatic compounds [138], which may then be taken up by the colon epithelium. Thus, a number of processes may occur in the upper and lower intestine, which have implications on the polyphenol profile, and therefore on the bioactivity profile, which often are disregarded in simplified *in vitro* trials.

2.3.3 Anti-oxidant aspects – direct effects

As many polyphenols can act as radical scavenging compounds and are thus able to act as antioxidants [16], their antioxidant potential has been thoroughly investigated *in vitro* and *in vivo*. Strongest antioxidants appear to be compounds with multiple hydroxyl groups, such as flavonoids or tannins [139]. However, bioavailability of many polyphenols may be low. Upon ingestion, polyphenols can be metabolized (deglucosylated, glucuronidated, sulphated, and possibly deesterified) by human enzymes, with additional changes in the gut by (typically, colonic) bacteria, which may hydrolyse glucosides,

glucuronides, sulfates, amides, esters, and lactones and further result in ring-fission, in addition to further reduction, decarboxylation, demethylation, and dehydroxylation, among other reactions [134, 136]. Polyphenols also are typically pumped rapidly out of the cell, often back into the gut lumen, via, for example, multidrug resistant proteins (MRP) 1,2,4,P-glycoprotein (P-gp), and breast cancer cell resistant proteins (BCRP), which further reduce their bioavailability. As, following absorption, also urinary excretion may occur rapidly, often following phase II metabolism (sulfation, glucuronidation), this finally results in quite low levels of detectable circulating polyphenols, especially native ones. In addition, as many other systems in the human body can act as radical quenching compounds, including enzymes such as SOD and GPx, other exogenous compounds (vitamins E and C, carotenoids), and many additional endogenous molecules (uric acid, albumin), the overall contribution to direct antioxidant effects therefore appears low [16].

However, polyphenols may have a role either via:

(a) their action as antioxidants prior to absorption, that is, directly in the gut lumen, where their concentration is comparatively high, quenching ROS occurring in the gut lumen ,or

(b) following absorption, via their influence on nuclear receptors and gene expression.

Unfortunately, not much is known about their possibility to quench ROS or reactive nitrogen species (RON) in the gut lumen prior to absorption, as this has never been systematically studied. As it is clear and has been demonstrated that the antioxidant potential of

polyphenols prevails, at least in part, during digestion, depending mostly on release kinetics and possible solubility, *i.e*. bioaccessibility, polyphenols can therefore contribute to antioxidant activity [16, 140] in the lumen of the gut. This may be important, as even for the extracellular space (*i.e*. gut lumen), ROS and RON may be released following inflammatory diseases into the gut, and quenching these species may reduce further aggravation of IBD conditions. However, the potential resulting health benefits in this respect have never been studied and may be more difficult to distinguish from effects following absorption and to extrapolate to the long-term effects *in vivo.*

2.3.4 Anti-oxidant aspects –effects via altering molecular targets

2.3.4.1 Animal trials

In contrast to effects prior to polyphenol uptake (*i.e*. their activity in the lumen), effects following their absorption and their influence on gene expression via molecular targets (e.g. transcription factors) have been investigated in more detail [141, 142], with mechanistic insights from animal and cellular models.

Table 4 gives an overview on frequently applied animal models. As can be seen, most studies have been finding positive effects based on intervention with various polyphenols and polyphenol rich sources, such as apples, green tea, cacao, pomegranate, and grape seeds, regarding the development of IBD, typically tested by "softmarkers," such as cytokine formation or other inflammation and oxidative stress related aspects, both locally and systemically, in conjunction with histological examinations. Regarding markers of

oxidative stress, polyphenols have been shown to modify the formation of MDA [87, 89, 91], hydrogen peroxide [98], protein oxidation [91], and several genes in the mucosa involved in antioxidant defence and detoxification, including, for example, glutathione peroxidase 1 (GPx-1), NAD(P)H dehydrogenase [quinone-1] (NQO-1), peroxiredoxin-6 (PRDX-6), superoxide dismutase 1 (SOD1), catalase (CAT), and thioredoxinreductase-1 (TXNRD1) [87, 95], in various rodent models, also confirmed in a study with healthy pigs receiving grapeseed and grapemarc extracts (1% in the diet) for 4 weeks, compared to control pigs. Mechanisms involved appeared to be related to the deactivation of further upstream targets, especially Nrf-2 [95], due to a high antioxidant effect of the extracts; at least this mechanism appears plausible. Often however, Nrf-2 is upregulated following higher doses of individual polyphenols, especially if oxidative stress levels are high. This has been corroborated by several studies, for example, in rats where gut inflammation and oxidative stress were induced by ketoprofen (nonsteroidal anti-inflammatory), receiving catechins (35 mg/kg per day) for 21 d, resulting in increased formation of Nrf-2 downstream targets, *i.e*. glutathione (GSH, reduced form), and also in reduced lactate dehydrogenase (LDH) leakage and 8-hydroxy-guanosine (8OHdG) [96].

The reduction of oxidative stress and inflammation in the gut has also been reported to have somewhat more systemic effects. In a study by Cazarin *et al.* [97], administration of *Passiflora edulis* peel rich in fiber and polyphenols for 7 d at 25 g/kg flour reduced serum antioxidant activity (FRAP), GPx, thiobarbituric acid reactive

substances such as MDA (TBARS), and glutathione reductase (GR). In another study with mice, green tea polyphenols given for 10 d (no dose specified) enhanced blood levels of GSH [85], and green tea polyphenols or EGCG (epigallocatechin gallate) at 0.25, 0.5, and 1 % added to the diet for10 weeks improved colonic and hepatic GSH in a similar model [58].

Typically, doses of polyphenols or extracts have been ranging between 10 and 20 mg/kg body weight of animals, though lower ones down to 0.5 mg/kg and higher ones up to 100 mg/kg or even 500 mg/kg for certain extracts have been administered (Table 3). Strictly up-scaling these concentrations to humans would result in doses of 700– 1400 mg, which is about the daily intake of polyphenols, being high, but achievable, surely with dietary supplements, while doses above would represent supra physiological amounts. When however taking into account body surface area (BSA), and applying the human equivalent dose (HED), the typical dose applied to animals would translate into approx. 190– 380 mg [143], given that the HED (mg/kg) equals animal dose (mg/kg) × (animal Km)/(human Km), with Km being a conversion factor, typically 57 for a human adult and 3 for a mouse (6 for a rat). However, it can be stated that most administered doses are indeed physiologically realistic and are within the daily human intake. Times of administration usually ranged from about 1 to 12 weeks or so, reflecting a considerable lifespan for small rodents.

2.3.4.2 Cell culture studies

The effects observed in animal models are generally confirmed by cell culture studies (Table 5). For example, following digestion of a

raspberry extract, the amount of ROS produced due to acrylamide, induced toxicity on Caco-2 cell mitochondria was significantly reduced [144]. More specifically, intracellular ROS generation was lowered, as was mitochondrial membrane potential (MMP) collapse as well as glutathione (GSH) depletion.

In a study applying red wine extract rich in catechin B1 and malvidin-3-glucose on HT-29 cells for 24h, both COX2 expression and protein tyrosine nitration, a biomarker of RON, were significantly reduced [69]. In another study, apple peel polyphenols (250μg/mL for 24h) reduced lipid peroxidation in Caco-2 cells [111]. At least some antioxidant effects observed *in vivo* may be ascribed to reduced neutrophil activity, which may produce several types of ROS, as shown by decreased production of ROS in neutrophils, following incubation with the ellagitannin metabolite urolithin B *in vitro* [145], possibly via inhibiting myeloperoxidase.

It has to be mentioned that some reports (though the minority of published results) did not confirm positive effects of polyphenols on Nrf-2 or downstream targets. In a study with grapeseed and grape-marc extracts rich in polyphenols, employing (TNF-α induced) Caco-2 cells exposed for 24h at 2mg/mL, no effects on Nrf-2 transactivation or target genes (GPx-2, NOQ1, CP1A1, and UGT1A1) were found [110]. In contrast, administration of a polyphenol-rich plum digesta to a Caco-2/HT-29 (apical) and THP-1 like macrophage (basolateral compartment) model (stimulated with a mixture of LPS, TNF-α, and IL-1 β) for 24 h even reduced transactivation of Nrf-2, possibly indicating reduced oxidative stress levels [67] following polyphenol exposure. These effects could well be concentration dependent, as it

has been suggested that certain antioxidants such as retinoic acid at high concentrations may trigger Nrf-2 translocation to the nucleus, while lower, more physiological concentrations, and perhaps employing stimuli not causing excessive oxidative stress responses, had no or even opposite effects [146], being in line with an overall reduced ROS level.

It can thus be speculated that at least in the epithelium (where concentrations of polyphenols may still be reasonably high compared to the deeper cell layers of the intestine) both direct antioxidant effects and more indirect effects, activating the cell's own antioxidant system, may play a role. Again, concentrations at the basolateral side are possibly lower due to the efflux of certain polyphenols by the mentioned transporters back into the lumen. However, it must also be stated that many studies have been applying relatively high doses of polyphenols and extracts to the epithelium, often 25 μM (e.g. ,resveratrol, *i.e*. ca. 6 mg/L) or even up to 100 mg/L for other compounds, reachable perhaps via supplements, but not easily with regular food items. It may be argued that, as more long-term effects can normally not be studied with monolayer cell culture models, higher concentrations may somewhat counterbalance for shorter exposure times (not considering additional model limitations such as missing digestion, form of application, *i.e*. bioaccessibility), though again (as mentioned above) effects of polyphenols may be well concentration dependent.

In conclusion, there is strong evidence from animal trials and *in vitro* (cellular) experiments that polyphenols, when applied in considerable, but still physiologically relevant doses, do reduce

oxidative stress in colonic epithelial cells and tissues stimulated for oxidative stress/inflammation and that the mechanism of oxidative stress is, at least in part related to the transcription factor Nrf-2, influencing further downstream targets.

Table 4. Animal trials suggesting positive health benefits of polyphenols with respect to IBD – an overview.

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of green tea PP on DSS induced colitis in IL-2 deficient mice	Water with 5g/L green tea PP for 6 weeks,	Reduced serum amyloid A, increased weight gain & hematocrit, reduced IFN-γ, TNF-α in cultured cells from colon	Anti-inflammatory effects of green tea PP	40
Effect of green tea PP and other antioxidants on DSS induced colitis in mice	10 d, no dose specified	Lengthening of colon, enhanced blood level of reduced GSH, improved serum amyloid A, TNF-α, improved cytoskeleton	Improved antioxidant status	41
Effect of ellagic acid (EA) on rats with TBNS induced colitis	10-11 rats per group receiving 10-20 mg/kg EA for 10 d	EA decreased neutrophil infiltration & COX-2 & iNOS. Reduced activation of p38, JNK & ERK1/2 MAPKs, preventing inhibitory protein IkB-degradation, inhibiting nuclear translocation of p65	EA diminished severity and extension of intestinal injuries. EA also increased mucus production in goblet c ells in colon mucosa	19
Effect of strawberry PP on rats with induced gastric lesions	40 mg/kg with various strawberries or quercetin (100 mg/kg) for 10 d (equiv. to 0.5 kg for 70 kg adult)	Reduced MDA, enhanced SOD and in part CAT in gastric mucosa.	Antioxidant enzyme activities increased with strawberry extract, decreased gastric lipid peroxidation. Sign. correlation between total anthocyanin content & % inhibition of ulcer	42
Effect of apple PP (APP) on mice with induced colitis	APP at 1% added to drinking water (90% tannins) for up to 4 weeks	APP administration dampened mRNA expression of IL-1β, TNF-α, IL-6, IL-17, IL-22, CXCL9, CXCL10, CXCL11, and IFN-γ in colon	APP-mediated protection required T cells. Giving APP during colitis to T-cell receptor(-/-) mice enhanced pro-inflammatory cytokine expression, showing need for TCRαβ cells in APP-mediated protection	43
Effect of ellagic acid (EA) and enriched pomegranate extract (PE) in TBNS induced rats	6 weeks with either 250 or 500 mg/kg PE, or 10 mg/kg EA or both together	MPO activity and TNF-α levels were significantly reduced in rats receiving PP	PE and an EA-enriched PE diets decreased COX-2 and iNOS expression, reduced MAPKs phosphorylation & prevented NF-κB translocation	19
Effect of EGCG and *Piper nigrum* on DSS induced colitis in mice	6.9 mg/kg bw. EGCG or *Piper nigrum* (2.9 mg/kg) for 60 d	Combination of EGCG & piperine sign. reduced loss of bw., improved clinical course and increased overall survival	Attenuated colitis was associated with reduced histological damage to colon & reduction of tissue concentrations of MDA. Neutrophil accumulation indicator MPO was reduced in the colon, SOD and GPx were increased	44

Table 4: Continued

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of green tea PP on DSS induced colitis in IL-10 deficient mice	Green tea PP or EGCG at 0.25, 0.5 and 1% added to diet for 10 weeks	Low dose improved histopathology, all doses improved antioxidant levels (colonic and hepatic GSH, reduction of circulating TNF-α & IL-6,	Antioxidant activities of polyphenols	14
Effect of grape juice on rats with TBNS induced colitis	1 or 2% grape juice in diet for up to 9d	1% grape juice improved clinical symptoms of colitis: reduced intensity of macroscopic and histological scores	Sig. differences of TNF-α and inducible NO synthase mRNA expression	45
Effect of oligonol (lychee PP) on mice with DSS induced colitis	0.5 or 5 mg/kg/d for 2 weeks	Oligonol sign. inhibited activation of NF-κB, STAT 3, COX-2, iNOS, & cyclin D1 in the colon. It also inhibited adenoma formation & attenuated MDA levels & protein oxidation (4-hydroxy-2-nonenal)	Various anti-inflammatory genes involved, as well as effects on antioxidant-status	46
Effects of *Phlomis purpurea L.* and *Phlomis lychnitis L.*on rats with DSS induced colitis	*P. lychnitis* (10 and 20 mg/kg), *P. purpurea* (10 and 25mg/kg) for 1 week	Both extracts reduced colonic MPO activity, increased colonic GSH, and down-regulated iNOS expression. Only *P. purpurea* extract reduced expression of IL-1β & IL-17, CINC-1 and MCP-1, and ICAM-1	Anti-inflammatory aspects of both extracts. Implication of NF-κB ?	47
Effect of green tea PP on Mdr1a(-/-) mice on proteomic and transcriptomic endpoints	0.6% in the diet for 12 weeks	Improved histopathology, reduced abundance of transcripts & proteins associated with immune & inflammatory response/fibrinogenesis, increased abundance of pathways associated with xenobiotic metabolism in response to GrTP	Anti-inflammatory activity mediated by multiple molecular pathways. PPAR-α and STAT1 appear to be key molecules regulating these effects	48
Effect of naringenin on DSS induced colitis mice	0.3% naringenin in diet for up to 9 d	Naringenin attenuated the increased DAI & colon shortening and suppressed the increased cytokine (IL-17A, IL-1ß, IL-6, MIP2 expression. Reduction of permeability	Anti-inflammatory properties of naringenin & barrier protection	49
Effect of grape seed and marc extract (GSME) on healthy pigs	GSME at 1% added to diet in 6 pigs vs. 6 control pigs for 4 weeks	Lower expression of NF-κB (ICAM-1, ccl-2, IL-8, TNF-α, SAA) and Nrf-2 (GPx-1, NQO-1, PRDX-6, SOD-1,TXNRD-1) target genes. No difference of conc. of plasma α -tocopherol & TBARS in liver & plasma & total antioxidant capacity	Pigs fed GSME diet had lower NF-κB and Nrf-2 transactivation in duodenal mucosa. Ratio of villus height:crypt depth & the gain:feed ratio was higher in pigs fed GSGME	50

Table 4: Continued

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of catechin, on rats receiving ketoprofen	Catechin (35 mg/kg/d) for 21 d	Catechin inhibited oxidative damage & reversed impairment of antioxidant system (GSH, LDH-leakage, 8-OHdG, in intestinal mucosa	ROS reduction of polyphenols	[51]
Passiflora edulis peel rich in fiber/PP on TBNS induced colitis in rats	7d, no PP conc. of passion fruit stated. 25 g passion fruit flour/kg diet given	Improved serum FRAP, GPx, TBARS, GR, decreased colon lipid peroxidation, decreased no. of aerobic bacteria & Enterobacteria, improved acetic & butyric acid levels in feces, higher no. of Bifidobacteria & *Lactobacilli*	Improved antioxidant status, improved gut flora	[52]
Effect of PP rich evening primrose pomace (EP) extract on TBNS induced colitis in mice	10 mg/kg of 612 mg/g PP extract per dry basis, for 3 d	Improved histopathology and MPO, reduced tissue hydrogen peroxide levels, no effect on IL-1ß , TNF-α	Reduced ROS via antioxidant activity	[53]
PP rich sorghum bran given to rats with DSS induced colitis	6% fiber diet given over 21 d	Diet significantly affected *Bacteroidales, Bacteroides, Clostridiales* and *Lactobacillus*	Protection via improving microbial diversity and richness, and dysbiosis of Firmicutes/ Bacteroidetes	[54]
Effect of grape seed PP in IL-10 deficient mice	16 weeks exposure with 1% GSP of dry food weight	Improved histopathology, reduced pore forming claudin-2 protein and increased barrier forming claudin-1 protein expression	Reduced expression of NF-κB, reduced beclin-1 and AMPK expression by GSE	[55]
Effects of grape seed PP (GSP) in DSS induced colitis in rats	21 d, 1.15 mg/g PP in diet	Reduced lesions (histological score) & disease activity index, reduced cytokines (IL-13, TNF-α, IL-1ß, IL-10, GM-CSF, IL-6, IL-1α, IF-γ), reduced MPO, enhanced GSH in colonic tissue	Up-regulation of various genes implicated in colitis such as intercellular adhesion molecule 1 (ICAM-1) and matrix metalloproteinase 9 (MMP-9)	[56]
Effects of gallic acid on induced colitis in mice	10 mg/kg for 7 d together with DSS	Improved histology scores, reduced TNF-α, IL-6, IL-1ß, IL-17, IFN-γ expression in colonic tissue	Reduced expression of p-Stat3, reduced expression of iNOS, COX-2, MPO in colon, reduction of p65-NF-κB	[57]
Effect of wheat anthocyanins on DSS induced colitis in mice	No dose specified. 14 d exposure	No sign. effects on colon length, bw, histopathology, markers of oxidative stress (FRAP, TAC, AOPP)	Degradation of anthocyanins, unclear dose, too much focus on anti-oxidant effects only	[58]

Table 4: Continued

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of cranberry extract on mice	C57BL/6J mice on high fat/high sucrose diet receiving either water or cranberry extract for 8 weeks	Mice receiving cranberry extract showed reduced intestinal oxidative stress and inflammation	Enhanced population with *Akkermansia* (mucus-degrading, SCFA producing), prebiotic effect	[59]
Effect of cacao extract on mice with DSS induced colitis	5 and 10% cacao diets for 62 d	Inhibited proliferation of tumor epithelial cells, suppressed colonic IL-6, TNF-α, IL-17, IL-1ß expression	Reduced expression and activation of STAT3, NF- κB, reduced expression of Bcl-xl, $CD68^+$ and MPO, enhanced caspase-3	[60]

See footnotes table 3.

Table 5. In-vitro trials suggesting health benefits of polyphenols with respect to IBD – an overview.

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of phytolens (water-soluble extract of PP antioxidants from non-soy legumes) on colonic (T84) & murine macrophage (RAW 264.7) cells	Phytolens (10-100 µM/ml) exposed to T84 & RAW 264.7 cells, 4 h & 12 h of exposure	Phytolens sign. attenuated apoptosis in T84 cells induced by ONOO-. Phytolens did not directly affect T84 cell viability or induce apoptosis after 4 h or overnight exposure. RAW 264.7 cells exposed to phytolens displayed decreased cell viability & increased apoptosis	Potential beneficial effects of phytolens on inflammation via attenuating induced apoptosis	61
PP standards effects of Caco-2 cells	Chrysin, ellagic acid, genistein & EGCG (all 50 µM) for 28 h	Chrysin and ellagic acid inhibited NF-κB activity, genistein and resveratrol increased it. Mixed effects on IL-8 secretion	Anti-and pro-inflammatory aspects of PP	62
Effect of PP metabolites 3,4-dihydroxyphenyl-acetic acid (ES) & 3-(3,4-dihydroxy-phenyl)-propionic acid (PS) on LT97 colon cells	Cells incubated with ES (0-18µM) & PS (0-90 µM), metabolites of quercetin & chlorogenic acid/ caffeic acid, respectively., up to 72 h	PP metabolites did not affect cell number but sign. upregulated GSTT2 expression and decreased COX-2	Intestinal metabolites showed anti-inflammatory properties	63
Effect of red-wine PP extract (from Lenoir grapes) on human colon-derived CCD-18Co myofibroblasts cells	0-100 µg/mL GAE, for 24 h	Red wine extract decreased mRNA expression of NF-κB, ICAM-1, VCAM-1, and PECAM-1, in a dose dependent manner	miR-126, a target region within the 3'-UTR of VCAM-1 mRNA, was increased at 100 µg/mL	64
Effect of PP-rich grape seed (GS) & grape marc (GM) on Caco-2 cells	24 h with up to 2 mg/mL	Decreased mRNA levels of NF-κB target genes IL-1β, IL-8, MCP-1 & CXCL1 in Caco-2 cells. Unchanged mRNA levels of Nrf-2 target genes GPX-2, NQO1, CYP1A1 & UGT1A1.	2 mg/mL ethanolic extracts dose dependently reduced NF-κB transactivation. No effect of ethanolic extracts was observed on Nrf-2	65
Effect of apple peel PP on Caco-2/15 cells	250 µg/mL apple peel PP for 24 h.	Apple peel PP prevented Fe/ascorbate-mediated lipid peroxidation & counteracted LPS-mediated inflammation by down-regulating cytokines (TNF-α, IL-6), & PGE-2	Down-regulation of COX-2 & NF-κB. Also induction of Nrf-2	66
Effect of red wine extract (RWE) (rich in catechin B1 and malvidin-3-glucoside) on HT-29 colon cells	100-600 µg/mL RWE extract (144 mg/g PP) for 24 h	RWE suppressed IκB degradation & IL-8 production dose-dependently. It also inhibited increase of NO from iNOS & of protein tyrosine nitration (biomarker of nitrosative stress)	RWE reduced NF-κB activation; COX-2 & iNOS.	25

Table 5: Continued

Aspects studied	Dosing and time	Effects found	Mechanism proposed and critics	Reference
Effect of catechin, theaflavin, malvidin, cyanidin & apigenin on human Int-407 cells treated with ketoprofen	25µM (malvidin, cyanidin & apigenin) or 100 µM (catechin & theaflavon for 5 h	Catechin sign. decreased levels of lipid peroxidation & ROS (MDA, DCF), & increased activity of intracellular antioxidant enzymes GPX, Gred, total sulfhydryl groups (TSH)	ROS reduction of PP. WB analysis revealed that catechin stimulated a time-dependent increase in Nrf-2 & total HO-1 protein expression	51
Effect of cyanidine-3-glucoside on HT-29 colon cells	25 µM cyanidine-3-glucoside, up to 24h	Improved NO, PGE-2 & IL-8 production,	Improved iNOS and COX-2 expressions. No effect on NF-κB or p38 MAPK, but STAT1	67
Effect of blueberry anthocyanin-rich extract on Caco-2 cells	Up to 100 µg/mL up to 48 h	Reduced IL-1β levels in Caco-2 cells	Reduced activation of NF-κB. 50 and 100 µg mL(-1) effective	68
Effect of resveratrol on HT-29 colon cells	25 µM resveratrol, up to 24 h	Decreasing levels of activated STAT1 in nucleus. Also reduction of cytokine-stimulated activation of SAPK/JNK pathway	Down-regulation of JAK-STAT pathway, though not counteracting cytokine-triggered negative feedback mechanism of STAT1 through p38 MAPK	69
Plum and cabbage digesta: Triple culture cell model: Caco-2/HT-29-MTX with THP-1	18 h of incubation following digestion	Reduction of IL-8 secretion by plum varieties	Influences via NF-κB & Nrf-2	24

See footnotes table

2.3.5 Anti-inflammatory aspects

2.3.5.1 Animal trials

While antioxidant effects surely do play a role in the origin and progression of IBD, more attention has been given to inflammatory aspects, possibly as a reduction in inflammation would likely also reduce ROS, and due to the meanwhile reasonably well understood molecular mechanisms underlying IBD, especially the involvement of NF-κB and its further downstream targets (Figure 1). However, also the JAK-STAT (janus kinase and signal transducer and activator of transcription) pathway may be involved, activated by interleukins/interferons, especially in cells of the immune system, resulting, for example, in the activation of apoptotic regulators, such as bcl-XL (B cell lymphoma extra-large, a transmembrane molecule in the mitochondria, acting as a prosurvival protein) of MYC (encoding for a nuclear phosphoprotein important for cell cycle progression and apoptosis), or alterations of the p21 antitumor progression gene [147]. A limited number of studies have included endpoints related to JAK/STAT. Barnett *et al.* reported anti-inflammatory activity mediated by multiple molecular pathways, including PPAR-α and STAT1, following administration of 0.6 % green tea polyphenols for 12 weeks to mice [93]. Lychee polyphenols (5mg/kg for 2 weeks) significantly reduced STAT3 activation in colon tissue of mice [91], and also adenoma inhibition was observed (Figure3).

In addition, the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway, where the phosphorylation of further downstream kinases can result in the activation of apoptosis or altered cell proliferation, is also implicated

in many inflammatory related diseases [146] and has been suggested to result in stimulated cytokine production by T-cells [148]. However, few studies have reported MAPK related effects following polyphenol administration. Some polyphenols have been reported to reduce MAPK related signalling pathways I in animal trials, including paeonol [149] and genistein [150], while a chalcone derivative [151] enhanced its activity. In a study by Rosillo *et al.* [86], raspberry polyphenols (10–20 mg/kg for 10 d) reduced activation of p38, c-JunN-terminal kinase (JNK), and ERK1/2 MAPKs, preventing inhibitory protein IκB-degradation, inhibiting nuclear translocation of p65 (part of NF-κB).

Many studies have meanwhile been performed on animal (typically rodent) models of IBD and intervention with various dietary components, including polyphenol-rich extracts, but also studies employing pure compounds (Table3). Of these pure compounds, particularly ellagic acid [63,86], gallic acid [102], naringenin [94], catechin [96],and EGCG [89] have been investigated and associated with anti-inflammatory effects. Thus, it appears that, with respect to anti-inflammatory properties, rather lower molecular weight polyphenols have attracted attention, as opposed to the more complex, that is, higher molecular ones that are regarded as potential prebiotics (see Section 3.5).

However, many extracts and complex food items rich in polyphenols have also been studied, with a focus on green tea [84, 85] and grape constituents [90, 95, 100], though many other food items, including strawberries [87], cranberries [104], *Pepper nigrum* [89], sorghum bran [99], and cacao [105], were also studied. With respect

to timing and dosing, polyphenol concentrations ranged from 0.5 to 100 mg/kg body weight for pure compounds, given over 3 days to 12 weeks, constituting high, but still physiologically relevant doses.

Both local effects and systemic effects on inflammation related pathways have been reported. Local effects did include decreased histopathological scores [58, 90, 100], improved length of the colon [94] as a marker of reduced severity of IBD and even reduced weight loss and improved overall survival [89] and inhibition of various cytokine formations such as TNF-α [84, 105], IL-6 [102], IL-10 [101], IL-17 [105], IL-1β [92, 105], IF-γ [101, 102], often linked to reduced expression of NF-κB [86, 91, 95], iNOS [90], and COX-2 [86, 102] (as a pre-step to the formation of proinflammatory prostaglandins, e.g., PGE-2) and peroxisome proliferator activated receptor(PPAR-) α [93], involved in lipid metabolism, in colonic tissues.

However, reduced cytokine levels in the circulatory system, including TNF-α and IL-6 [58], have also been found. Thus, results are in agreement with the theory that polyphenols or their degradation products/metabolites (cleaved aglycones, or glucuronidated and sulphated products) do act on intracellular signaling cascades in the epithelium or in infiltrated immune cells such as neutrophils [86] in the gut, downregulating proinflammatory cytokines, besides the likelihood that the majority of polyphenols is reexcreted into the gut lumen [30]. Whether the systemic measured effects reflect mostly cytokines secreted at the site of the gut, or whether polyphenols also pose considerably anti-inflammatory effects at different sites, is not entirely clear. Since polyphenols have also been reported to reduce

inflammation in other chronic inflammation related diseases, such as diabetes [152], both at least appear to be possible. In a study by Skyberg *et al.* [88], it was also verified whether polyphenols, when given peritoneal, would decrease induced colitis in mice. Contrarily to apple polyphenols given orally, no positive effects however were found, highlighting the importance of oral uptake and direct contact of polyphenols to the epithelial cells, and/or perhaps the prerequisite of forming certain degradation products or metabolites during digestion and/or at the epithelial layer.

The study by Skyberg *et al.* [88] also highlighted the involvement of T-cells (chemokine receptor CXCR3 expressing TCR$\alpha\beta$ cells) for apple polyphenol mediated protection, as these cells were indispensable for offering protection against colitis. Previous *in vitro* studies had already suggested that polyphenols can stimulate natural killer cells and $\gamma\delta$ T cells (typically found in high abundance in the mucosa), as these cells can upregulate CD69, CD11b, and IL2R proliferation and induce proinflammatory mRNA transcripts [88]. Thus, also specific immunomodulatory aspects of polyphenols should not be overlooked but have so far received comparably little attention. This is also underpinned by studies showing positive effects of polyphenols on toll-like receptors (TLRs), enhancing the activity of the innate immune system [153]. Also, the excretion of IgA in rats fed with extracts of haskap (honeyberry) and aronia fruits was increased, likewise demonstrating immune-stimulating effects [154].

2.3.5.2 Cell culture investigations

Though cell culture studies suffer various drawbacks, such as allowing studying inflammatory processes only during relatively short-term

periods, and often lack the complexity of the *in vivo* epithelium, *i.e*. the interactions between the various cell types involved in inflammation of the intestine, it is possible to examine many factors in rather short time periods, yielding mechanistic insights into the relation of dietary compounds and inflammatory processes, with or without preceding digestion.

Employing a triple culture model (with Caco-2/HT-29 MTX cells in the apical and THP-1 like macrophages in the basolateral compartment) coupled to preceding simulated gastrointestinal digestion, Kaulmann *et al.* [67] reported that digested plum extracts (ca. 1.2 g/L wet weight) were able to reduce IL-8 production by Caco-2/HT-29 cells, and several kale extracts reduced IL-6 secretion in THP-1cells, which was related to reduced NF-κB expression. However, both extracts rich and poor in polyphenols (and in carotenoids) did exert positive effects, suggesting that other compounds at least contributed to the positive effects, such as vitamin C. Romier *et al.* [107] investigated a variety of polyphenols and extracts with respect to inflammatory endpoints when exposed to Caco-2 cells, finding somewhat ambivalent results. While chrysin and ellagic acid (50 μM) reduced NF-κB expression, resveratrol and genistein increased it. Chrysin, ellagic acid, genistein, and epigallocatechin gallate reduced IL-8 secretion, while again resveratrol promoted it, pointing out that some polyphenols may show arbitrary effects when ingested at high concentrations. However, polyphenols did, as in most studies, not undergo simulated digestion, which on the other hand may not have had drastic effects on the compounds investigated, as these were mostly water soluble (not

requiring solubilisation in form of micelles, except perhaps resveratrol) and were administered as aglycones.

Another limitation is that colonic fermentation is usually not coupled to *in vitro* trials, though possibly strongly affecting polyphenol profile. The difficulty rests again in the noncompatibility of the epithelial cells employed and the bacteria, plus the difficulties to maintain strict anaerobic conditions. Also, very few studies have included colonic metabolites. Miene *et al.* [108] investigated the effect of quercetin and chlorogenic acid/caffeic acid metabolites (3,4-dihydroxyphenylacetic acid (ES) and 3-(3,4-dihydroxyphenyl)-propionic acid (PS), resp.) on colonic LT97 cells, finding reduced COX-2 expression. A further difficulty rests in the fact that many of the colonic metabolites are not commercially available and therefore remain understudied.

Most cell culture studies have been conducted with pure polyphenols, including especially curcumin, resveratrol [114], genistein, chrysin, and EGCG [107], cyanidin-3-glucoside [112], and catechin, theaflavin, malvidin, cyanidin, and apigenin [96], though extracts, especially red wine [69, 109], apple (peel) [111], blueberry [113], and grape [110], have also been studied. Concentrations of individual compounds, as stated also above, have ranged from ca. 25 to 100 μM, and of extracts up to 600 μg/mL, which is considered high but physiologically reachable in the gut. A drawback of most studies, again, is the missing preceding digestion, which would limit especially the bioavailability of the more apolar polyphenols, namely, resveratrol and curcumin, due to missing emulsification, that is, solubilisation in mixed micelles. Most models have included Caco-2

cells or HT-29 cells, which may underestimate the strength of *in vivo* responses, as immune cells have mostly not been employed. The majority of these trials have demonstrated that polyphenols or polyphenol-rich extracts were able to reduce proinflammatory cytokines, including typically IL-8 [67,69,107,110,112], but also PGE-2, TNF-α and IL-1β, often both at mRNA expression and at protein level, and that this was related to reduced NF-κB expression (Table5).

Other studies have focussed on downstream targets of COX-2. In a study by Serra *et al.* [112], cyanidin-3-glucose administered for 24 h at 25 μM reduced PGE-2 expression in HT-29 cells, possibly as a consequence of influencing COX-2 expression, which was also detected. Apple peel polyphenols (250 μg/mL) for 24h also reduced COX-2 activity [111] in Caco-2cells.

The fact that also intracellular adhesion molecule (ICAM-) 1 was significantly downregulated by, for example, red wine polyphenols [109], important for leukocyte endothelial transmigration, monocyte chemoattractant protein 1 (MCP-1), and chemokine (C-X-C motif) ligand 1 (CXCL1), also having neutrophil chemoattractant activity, vascular cell adhesion protein-1(VCAM-1, promoting adhesion of other immune cells), and platelet endothelial cell adhesion molecule 1 (PECAM-1, equal to CD31, playing a role in neutrophil removal), in further studies (Table 4), also suggests, as do animal studies, that modulation of the immune system is also a potential important function of polyphenols. Furthermore, apoptosis may also be influenced, as shown with soy legume extracts on both T84 colon cells and macrophages at over 30 μM [106], where inflammation

related apoptosis in the epithelial cells (induced by peroxynitrites) was significantly reduced, while macrophage viability was compromised.

In conclusion, several cellular mechanistic studies are in line with animal study findings that both polyphenols and polyphenol-rich products are able to reduce the concentration of proinflammatory cytokines, acting via reduced NF-κB expression and translocation, though additional functions such as modulation of the immune system, reducing, for example, leukocyte transmigration, neutrophil attraction, and finally altered apoptosis, may also play a role.

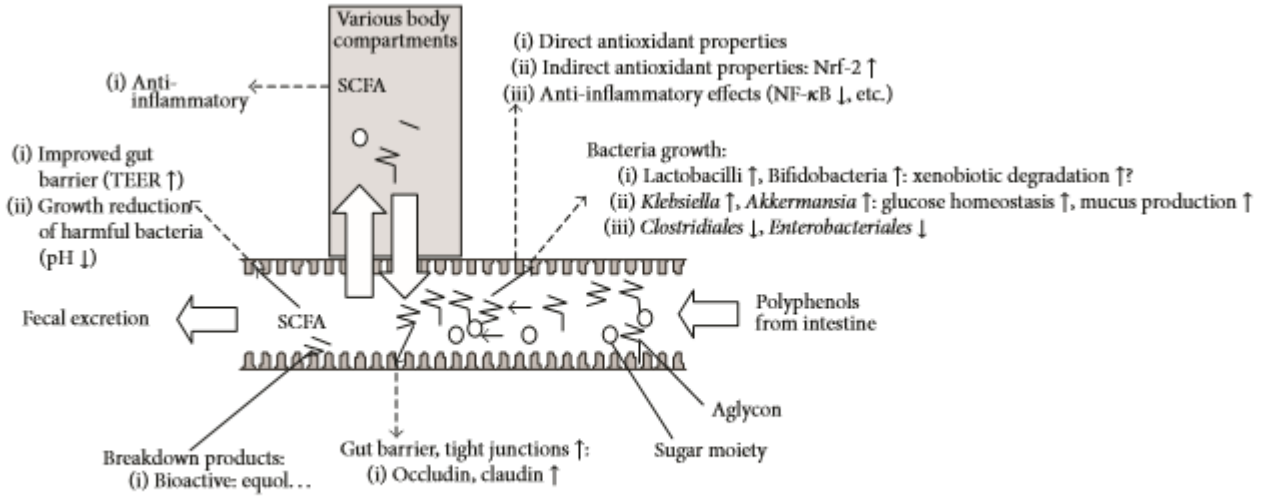

FIGURE 3: Potential effects of polyphenols on the gut microbiota and possible resulting health effects. SCFA: short-chain fatty acid (butyrate, propionate, and acetate). TEER: trans-epithelial electrical resistance.

2.3.6 Gut microflora

The importance of the gut microbiota has recently been highlighted in a study by Schaubeck *et al.* [155], where disease associated microbiota was transplanted in a mouse model, causing CD in the transfected mice, clearly demonstrating gut microflora as a causative agent of IBD.

Several studies including cellular, animal, and human trials have indicated that polyphenols from fruits or vegetables may increase the number of potential health beneficial bacteria in the gut [156–158], thus acting as prebiotics. This in turn is expected to have positive intestinal effects, including, for example, the formation of SCFA, to enhance gut barrier properties, and fostering the growth of potentially less harmful bacteria. Earlier trials in this respect have shown, for instance, that, in Caco-2 cells, propionate, acetate, and especially butyrate (2mM) increased the transepithelial electrical resistance (TEER) by almost 300 % [159] after 72 h of incubation with these SCFAs. As however also DMSO showed similar effects, it was reasoned that further cell differentiation played a major role in reducing permeability. On the other hand, germ-free mice do not develop IBD, and humans treated with antibiotics appear to result in at least temporary remission of IBD [15], emphasizing that the microbiota could act as a double edged sword.

Nevertheless, the question to what extent and via which mechanisms polyphenols may act as prebiotics is only poorly comprehended, though the evidence that polyphenols contribute to the number of health beneficial bacteria such as *Lactobacilli* and

Bifidobacteria is increasing [158,160], while suggesting a likewise reduction in orders including potential pathogenic bacteria such as *Clostridiales* and *Enterobacteriales*, and even LPS or toxin producing *E.coli* strains, such as O157:H7 [161]. It has also been suggested, based on *in vitro* studies, that the effects are rather related to the aglycones than the glycosides, at least for flavonoids [162], perhaps also emphasizing the role that bacteria could play in deglycosylation of the native glycosides. Altering the gut flora can have percussions on the further degradation of carbohydrates, including potential probiotics. In an *in vitro* study by Xue *et al.*[163], the plant polyphenols quercetin, catechin, and puerarin, when added to the cellular media, downregulated the ratio of Firmicutes: Bacteroidetes, which altered the degradation of fructo oligosaccharides (FOS). Similarly, in a study with anthocyanin rich strawberry extract [164], the formation of FOS breakdown products and acidification achieved in the gut was increased in rat cecal digesta and urine, also emphasizing the potential positive effect of additional polyphenols and their implication in energy metabolism. In addition to their influence on carbohydrate metabolism, polyphenols have also been reported to influence lipid and provitamin metabolism, via their alteration of gut microbiota, and therefore could also change human homeostasis [165,166].

Several *in vitro* studies have pointed out that polyphenol-rich extracts, such as from pomegranate, can enhance the growth of *Bifidobacterium* and *Lactobacillus* [167]. Similar effects in a batch-culture fermentation *in vitro* model were observed with coffee with high levels of chlorogenic acid [168]. Studies in this domain have

been impeded by the fact that many bacteria cannot easily be grown *ex vivo*, though models simulating also colonic digestion under realistic, *i.e*. low oxygen, environmental conditions, such as the SHIME, have further deepened the understanding of the interrelation between the diet and the microbiota [169], also highlighting that polyphenols can provoke a shift of the microbiota. This included, for example, the growth of *Klebsiella* and *Akkermansia* spp., with the latter growing especially in mucus-rich environment, producing SCFA. Particularly proanthocyanidins, oligomeric flavonoids, rich, for example, in barks of trees but also in grapes, have been speculated to foster the growth of *Akkermansia* [160], as it increased mucus layer thickness, improved glucose homeostasis, and alleviated metabolic endotoxemia. An additional effect may rest in the influence *Akkermansia* appears to exert on branched chain aminoacids (BCAA). A low plasma level of BCAA has been associated with reduced insulin secretion and lower weight in obese subjects [170]; however, they may also promote a more systemic proinflammatory response[171].

In addition to causing a shift toward an altered microbiota composition, it appears that polyphenols also induce a transformation in bacterial genes toward xenobiotic degradation, as found in a study on rats receiving blueberry powder for 6 weeks, whereas genes related to BCAA degradation, known to reduce gut atrophy [172], and those associated with higher invasiveness were enhanced and reduced, respectively [161], thus perhaps fostering the deactivation of other harmful xenobiotic compounds and possibly improving gut barrier function.

A few *in vivo* studies, including animals and humans (see Sections 2 and 3), have confirmed the positive effects that polyphenol-rich extracts may exert toward fostering healthy microflora. In pigs, proanthocyanidins in grapeseed extract (1 %) consumed over 6 d enhanced however *Lachnospiraceae*, *Clostridiales*, *Lactobacillus*, and *Ruminococcaceae* [173], also showing that effects are surely model and polyphenol dependent. C57BL/6J mice kept on a high fat/high sucrose diet receiving either water or cranberry extract (200mg/kg) for 8weeks showed an enhanced population of *Akkermansia*, in addition to reducing intestinal oxidative stress and inflammation [104]. Similar effects (enhanced Bacteroidetes/Firmicutes ratio, often associated with less obese subjects; reduced *Bacillus*; increased *Akkermansia muciniphila*) were also observed when giving resveratrol (15 mg/kg bw.) or quercetin (30 mg/kg bw.) to rats for 6 weeks [174], highlighting the effects of pure polyphenols not associated with dietary fiber. Similarly, administration of a carbohydrate-free plum or peach extract (430 and 1,270 mg GAE (gallic acid equivalents)/mL, resp.) to obese sugar rats for 11 weeks resulted in increased abundance of *Lactobacillus* and members of *Ruminococcaceae*, especially in the more polyphenol-rich plum extract.

In conclusion, several *in vitro*, animal, and even human trials have suggested that polyphenols, in physiological doses, that is, achievable via a diet rich in fruits and vegetables, or with supplements, over the time-course of a few weeks, are able to shift the microbiota toward a more presumably healthy one, with an enhanced population of Bacteroidetes, producing SCFA, and perhaps being capable of a faster degradation of certain xenobiotic compounds.

2.3.7 Epithelium protection and other aspects

In addition to strengthening intestinal barrier properties via fostering the growth of health beneficial bacteria (see Section 3.6), polyphenols have further been proposed to influence directly the permeability of the mucosa, via acting on the tight junctions, composed especially of occluding and claudin proteins. In a study by Carrasco-Pozoetal. [175], several polyphenols, that is, quercetin, epigallocatechin gallate, and resveratrol (though not rutin), protected tight junction integrity in a Caco-2 based model, via inhibiting the redistribution of the zonula occludens-(ZO-)1 protein induced by indomethacin and preventing the decreased expression of ZO-1 and occludin caused by indomethacin, possibly related to the polyphenol capacity to protect the mitochondria and reduce ATP depletion. Similarly, in a study employing Ussing chambers and T84 monolayers, polyphenols (ferulic and isoferulic acid, but not caffeic or p-coumaric acid) reversed the negative effect of sodium caprate on tight junction functionality [176], as measured by TEER. This effect was ascribed to the increased expression of tight junction components of ZO1 and claudin-4 transcription and reduced occludin expression. In a similar study employing T84 cell monolayers, the negative effects of sodium caprate on tight junction associated genes were counterbalanced by several apple polyphenols and their presumed intestinal digestion products, including caffeate, quinic acid, and methyl-p-coumarate [177]. In a study with Caco-2 cells, quercetin (and its metabolite 3,4dihydroxybenzoic acid) enhanced epithelial resistance to 157 (and 119 %, resp.), of control TEER values, which was related to an increased expression rate of claudin-4 [178]. Positive effects on cellular barriers *in vitro* were also

found following cayenne pepper and paprika exposure (reviewed by [179]), though these may have contained considerable amounts of other bioactive compounds, such as vitamin C and carotenoids.

However, also negative effects on tight junctions have been reported, for example, when giving ochratoxin together with polyphenols from (dealcoholised) red wine, via enhanced intracellular redistribution of claudin-4 [180], perhaps as a result of increased uptake and/or reduced excretion and/or metabolism of ochratoxin in the presence of polyphenols.

These, at least in part, positive findings of polyphenols are corroborated by animal studies. In a study by Yang *et al.* [100], grape seed polyphenols (1 % dry weight added to diet given for 16 weeks) improved claudin-2 protein and increased barrier forming claudin-1 protein expression in IL-10 deficient mice, occasionally employed as a model of IBD. Reduced colonic permeability as measured in everted colons by a fluorescein dye was found in colitis induced mice receiving 0.3 % naringenin for 9d in the diet [94]. In a study on rats, curcumin was able to protect the nephron from the negative effects of cisplatin, an anticancer drug, improving stability of tight junctions via enhancing the expression of adherens junction proteins occludin, claudin-2, and E cadherin [181]. Likewise, in a study with rats investigating neuroprotective effects, subjected to occlusion of the cerebral artery, receiving green tea polyphenols (400 mg/kg and day for 30 d), decreased mRNA/protein expressions of claudin-5, occludin, and ZO-1 in microvessels of ischemic tissue were prevented [182]. Thus, taken together, the results suggest that polyphenols can improve gene expression related to the production of proteins required

for tight junction integrity, including possibly claudin-5, occludin, and ZO-1, and that these effects may not be limited to the gut epithelium only.

An additional effect of barrier protection may also rest in increased mucus production or a more stable mucus layer. As also the mucus may have an important barrier and protection function, limiting direct contact of potential proinflammatory stimuli with the epithelial cells, mucus production was likewise investigated in several studies. Rosillo *et al.* [86] scrutinized the effect of ellagic acid (10–20 mg/kg) in a rat model with TBNS induced colitis. Among others, enhanced mucus production by goblet cells in the colon mucosa was found. Likewise, B-proanthocyanidin-rich extracts were suggested to increase mucus secretion, in turn creating a suitable environment for *Akkermansia*, which may further foster SCFA production [80]. Dietary polyphenols have also been stated to be able to cross-link mucin, enhancing the viscoelastic modulus of the mucus layer [183], stabilizing the mucus layer in the intestine [184]. Nevertheless, more studies in this domain are required.

2.4 Potential arbitrary effects of polyphenols or absence of effects

With respect to at least IBD, despite the fact that there is much evidence accumulating emphasizing the potential health benefits of polyphenols, it should not be overlooked that polyphenols may also have arbitrary effects on the gut epithelium and the host in general. In

addition, there is still a general paucity with respect to robust human trials (*i.e*. placebo controlled randomized intervention trials) clearly demonstrating positive health effects of polyphenols regarding IBD development and/or progression. In addition, several studies have also cast some doubts that polyphenols, at least alone, are truly the (sole) bioactive agents. For example, in a study with plums and cabbages with contrasting polyphenol and carotenoid profile, concentrations of these phytochemicals were not related to higher anti-inflammatory bioactivity in an *in vitro* model of digestion and inflammation, suggesting that other bioactive compounds, including dietary fiber or vitamin C, may also contribute to the observed effects regarding, for example, cytokine (IL8, IL-6) reduction, NF-κB, and Nrf-2 translocation [67]. Finally, it should also be considered that many trials have been conducted *in vitro* with native components, without preceding digestion and colonic fermentation, which are not likely to be present in the colon under *in vivo* conditions, and not at the rather high concentrations often employed. Furthermore, polyphenols may, in response to dose and nature, enact negative effects via the following pathways:

(a) They could act as prooxidants, especially when given isolated and in high doses.
(b) They may perturb absorption of other bioactive compounds, such as drugs or other phytochemicals.
(c) They may interact and/or saturate pathways related to phase I/II metabolism, likewise increasing the concentration of otherwise more highly metabolised bioactive compounds.

(d) They may have other negative effects following bacterial metabolism.

With respect to acting as prooxidants, it has been highlighted that especially compounds with several free hydroxyl groups, such as flavonoids, can, in the presence of free metal ions such as copper or iron, released, for example, when tissue is damaged (expected in subjects with IBD), act as prooxidants, via the Fenton reaction (producing peroxides), as reviewed previously [16, 185]. For some compounds, such as for quercetin, adverse effects at higher concentrations have indeed been shown, especially if reduced glutathione is already low and ROS level already high, as the oxidized quercetin-quinone product will then react with other thiol groups (e.g., from enzymes) [186]. Rat feeding experiments with quercetin have corroborated these results, showing decreased hepatic glutathione concentration and glutathione reductase when receiving 20 mg quercetin/day for up to 6 weeks [187]. Thus, type and dosing of polyphenols should be carefully considered for subjects already showing oxidative stress, such as for smokers, but may be also for IBD patients. It should also not be overlooked that the administration of other antioxidants, such as beta-carotene, though being much more apolar, has been suggested to cause detrimental health effects in human meta-analyses [188].

As stated, it has to be considered that polyphenols may also block certain efflux transporters in the gut epithelium [30] and may increase the uptake of toxicological relevant compounds, therefore constituting a double edged sword. For example, in a study with Caco-2 cells, dealcoholized red wine aggravated the permeability of the

monolayer when ochratoxin was also given [180], even though a recent study on rats did not detect significantly altered toxicokinetics in rats receiving both ochratoxin and quercetin [189]. The potential interactions between secondary plant compounds and other xenobiotics have been highlighted in an earlier review [190].

Similarly, phenol rich matrices such as grapefruit juice or green tea extracts have been hypothesized, due to their high content of polyphenols (naringenin and catechins, resp.), to reduce certain phase I metabolising enzymes (e.g., cytochrome P-450 (CYP) 3A4 isoenzyme), increasing the concentrations of unmetabolized drugs such as statins or antihistamines [191, 192], though they may in addition also reduce efflux-transporter activity such as P-gp, likewise increasing their apparent (absorbed) dose. The same mechanism has been suggested to contribute to high bioavailability of curcuminoids when piperidine (present in, e.g., black pepper) is simultaneously administered [193].

Finally, bacterial metabolites of polyphenols have been reported to also have potential negative effects. Following quercetin and rutin metabolism, the produced 3,4 dihydrophenylacetic acid (DOPAC), which was reported to show anticancer and anti-inflammatory properties, may also inhibit mitochondrial respiration, though this has been rather shown for brain mitochondria [156], nevertheless also suggesting that the therapeutic window may be limited, and that higher concentrations of certain compounds or metabolites could cause enhanced cellular damage. Other compounds, including resveratrol and genistein, have, when administered to cell

models at high but physiological concentrations (50 μM), enhanced NF-κB expression, suggesting proinflammatory behaviour

2.5 Conclusions

Many animal and *in vitro* (cellular) experiments have shown and emphasized positive effects of polyphenol-rich plants, their extracts, and also individual compounds, on ameliorating the severity and progression of IBD. It appears that polyphenols may not be the sole constituents with health beneficial properties in extracts or more complex matrices, but that other compounds, such as dietary fiber, or vitamin C, may also have certain effects.

Nevertheless, it appears likely that, by themselves, polyphenols can exert positive effects, reducing oxidative stress caused by or aggravated by infiltrating neutrophils and macrophages, and are able to locally reduce inflammation, most likely via acting on molecular targets such as NFκB (related to inflammation) and Nrf2 (related to oxidative stress), with the latter mechanism requiring cellular uptake into the epithelium. In addition, several studies have suggested prebiotic like effects, fostering the growth of healthy microflora (e.g. *Bacteroidetes*),which may have anti-inflammatory effects, for example, via SCFA production, or aiding in stabilizing barrier properties, which may also occur via direct effects on claudins and occludins or, alternatively, on the mucus layer. Nevertheless, administering high doses of polyphenols may also pose a certain risk to subjects already suffering from oxidative stress and inflammation, as polyphenols could also act as prooxidants, perhaps especially when

administered in high individual doses. Finally, due to their efflux-altering properties and effects on various CYP metabolising enzymes, interactions with drugs and other xenobiotics should be carefully considered. In addition to more sophisticated cellular models and enhanced commercial availability of colonic metabolites, more human trials are needed to confirm that polyphenols could in fact constitute a preventive strategy and/or supplementary treatment for subjects suffering from IBD and whether individual polyphenols or rather complex mixtures such as extracts are more potent and promising in order to ameliorate this ailment.

Competing Interests

The authors declare no conflict of interests.

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Carotenoids, Inflammation, and Oxidative Stress– Implications of Cellular Signaling Pathways and Relation to Chronic Disease Prevention

Abstract:

Several epidemiologic studies have shown that diets rich in fruits and vegetables reduce the risk of developing several chronic diseases, such as type 2 diabetes, atherosclerosis, and cancer. These diseases are linked with systemic, low-grade chronic inflammation. Although controversy persists on the bioactive ingredients, several secondary plant metabolites have been associated with these beneficial health effects. Carotenoids represent the most abundant lipid-soluble phytochemicals, and *in vitro* and *in vivo* studies have suggested that they have antioxidant, antiapoptotic, and anti-inflammatory properties. Recently, many of these properties have been linked to the effect of carotenoids on intracellular signaling cascades, thereby influencing gene expression and protein translation. By blocking the translocation of nuclear factor κB to the nucleus, carotenoids are able to interact with this signalling pathway and thus inhibit the downstream production of inflammatory cytokines, such as interleukin-8 or prostaglandin E2. Carotenoids can also block oxidative stress by interacting with the nuclear factor erythroid 2– related factor 2 pathway, enhancing its translocation into the nucleus, and activating phase II enzymes and antioxidant enzymes, such as glutathione-S-transferases. In this review, which is organized into *in vitro*, animal, and human investigations, we summarized current knowledge on carotenoids and metabolites with respect to their ability to modulate inflammatory and oxidative stress pathways and discuss potential dose-health relations. Although many pathways involved in the bioactivity of carotenoids have been revealed, future research should

be directed toward dose-response relations of carotenoids, their metabolites, and their effect on transcription factors and metabolism.

3.1 Introduction: carotenoids as antioxidants

Many nutrition and health organizations recommend regular consumption of fruits and vegetables because it is supposed to decrease the incidence of several chronic diseases such as type 2 diabetes [1,2], cardiovascular diseases (CVDs) [3] such as atherosclerosis [4], and several types of cancer [5–7]. These chronic diseases are associated with a systemic, low-grade chronic inflammatory component that is characterized by elevated circulating inflammatory markers such as cytokines (e.g. interleukin [IL]-8, IL-6, IL-1, IL-12) [8–10]; other inflammatory stimulating compounds such as prostaglandin E2 (PGE-2) [11], tumor necrosis factor α (TNF-α) [10], and interferons [10]; acute-phase proteins such as C-reactive protein [12,13]; immune cells associated with inflammatory responses such as macrophages [14] or eosinophiles [15]; and elevated markers of oxidative stress, for example, prostaglandins [16], isoprostanes [17], oxidized cholesterol [18], or oxidized lipid compounds such as malondialdehyde (MDA) [19]. These factors may result in additional tissue damage [20] and eventually aggravate disease.

Despite other potential dietary confounding factors such as vitamin C, vitamin E, or dietary fiber, several studies have attributed observed beneficial health effects to the consumption of secondary plant compounds such as polyphenols [21,22] and carotenoids [23,24]. Among those, carotenoids reach the highest plasma and tissue concentrations, ca. 2 μM [25], despite their lower intake compared with, for example, polyphenols [26]. The most abundant carotenoids in plasma include lycopene, β-carotene, and lutein [25]. In addition,

their plasma half-life is relatively long (days to weeks compared with 2-30 hours for polyphenols) because of their fat solubility, limited phase II metabolism, and decreased renal clearance [27–29]. Carotenoid consumption and tissue levels have been related to the prevention of cancer [30,31], diabetes [1,23,32], and inflammatory bowel diseases [33,34].

Carotenoids are liposoluble C-40–based isoprenoid pigments. They are characterized by an extended conjugated π-electron system that can only be synthesized by plants and microorganisms [35]. Animals, including humans, must rely on dietary uptake. To date, approximately 700 different carotenoid species have been identified, but only 50 have been reported to play a role in the human diet [36], with an intake of ca. 5-15 mg/d *per capita* [37]. Carotenoids can be separated into the oxygen-devoid carotenes and the oxygen-containing xanthophylls [3]. They can be further classified into provitamin A carotenoids (e.g. β-carotene and β-cryptoxanthin) and the non–provitamin A carotenoids, which cannot be converted to retinal (eg, lycopene and lutein) [38].

The extended π-electron system is an important feature of carotenoids because it aids in stabilizing unpaired electrons after radical quenching [39]. Because of this conjugated double-bond structure, carotenoids are strong scavengers of singlet oxygen ($^1O2$) and peroxyl radicals [40]. They either act via physical quenching, electron acceptance, or donation [41] or via hydrogen abstraction/acceptance [42]. Singlet oxygen scavenging by carotenoids depends largely on physical quenching, that is, a direct energy transfer between the 2 molecules. This scavenging depends on the number of conjugated double bonds [43]. Thus, carotenoids with more extended π-electron

systems, such as lycopene, are generally reported to constitute stronger antioxidants compared with phytoene/phytofluene [44].

The carotenoids also play an important role in their orientation within biological membranes [45]. As lipid-soluble molecules, different carotenoid structures are found in lipophilic environments and lipid/water interfaces. Xanthophylls, which are less hydrophobic than carotenes, are found in cellular membranes at the lipid/aqueous interface, and they can scavenge lipid and aqueous phase radicals [41]. Carotenes scavenge radicals in the lipid phase, as they are mostly located deep in the apolar core of lipid membranes [46]. Thus, within cells, carotenoids are affiliated with various types of membranes, such as the outer cell membrane, but also the mitochondria and the nucleus [47]. They also can be found in liposomes [48], whereas their free occurrence in the cytosol is rather low [47]. As a consequence, carotenoids play an important role in protecting cellular membranes [49] and lipoproteins [50] against damage by peroxyl radicals.

In addition to their scavenging function toward several reactive oxygen species (ROS), there is growing awareness that carotenoids may also act via more indirect pathways. This indirect route may include interactions with cellular signaling cascades, such as nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK), or nuclear factor erythroid 2–related factor 2 (Nrf2) [51,52]. Because of their rather low tissue concentrations and the regulation of antioxidant balance by numerous other endogenous compounds, the capacity for scavenging radicals is of biological relevance. After their cellular uptake from mixed micelles and the resulting symmetric (β-carotene-oxygenase 1, or BCO1) and assymetric apocarotenoids (β-carotene-di-

oxygenase 2, BCDO2, their activity depending on genetic factors and the administered dosage [53]), appear to be bioactive.

Presenting the findings from *in vitro*, animal, and human investigations, this review aims at summarizing current knowledge on the part carotenoids may play in inhibiting inflammatory and oxidative stress related processes by interacting with cellular signaling cascades. Search criteria in PubMed included the terms “carotenoids” combined with one of the following: “inflammation,” “oxidative stress,” “meta-analyses,” “NF-κB,” “reactive oxygen species,” “MAPK,” “animal studies,” or “Nrf2”; the results were then further filtered manually. The literature was searched from inception of PubMed until the present (2014). The initial search yielded 1657 potential studies, with 215 of these selected for this review.

3.2 Oxidative stress, inflammation, and intracellular signaling cascades

Inflammation, under normal conditions, is a protective mechanism of tissues against endogenous and exogenous damage [54]. Several agents and conditions that could lead to inflammation are known, such as microbial and viral infections, autoimmune diseases, exposure to allergens or toxic chemicals, and even metabolic disturbances that include obesity [55]. Two stages of inflammation are distinguished, the acute and chronic phases. Acute inflammation is the initial stage that persists for only a short time, and it is normally beneficial to the host because it helps to reestablish normal homeostasis, for example by digesting foreign bacteria. However, if persisting over a prolonged period, this state is referred to as chronic inflammation. This is

harmful for the body because it can result in abnormal physiological responses, increase the risk of cellular damage, and lead to the development of chronic diseases, such as cancer [55].

During inflammation, cells of the immune system such as macrophages and leucocytes are recruited to the site of damage. This results in a "respiratory burst," an overproduction of ROS, and leads to oxidative stress and damage of important biomolecules, such as proteins or DNA [54]. The inflamed cells also produce soluble mediators such as cytokines, chemokines, and metabolites of arachidonic acid (*i.e*. prostaglandins), which further recruit macrophages and are important key activators of different signal transduction cascades and transcription factors, such as NF-κB or Nrf2 (Figure). The activation of these cascades and transcription factors then results in the production and secretion of cellular stress responses such as cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), chemokines, and cytokines [55]. Transcription factors, specifically NF-κB and Nrf2, have been associated with inflammation and oxidative stress responses, respectively.

Nuclear factor κB is responsible for the transcription of a variety of genes that regulate inflammatory responses. When cells are not stimulated, NF-κB is bound to its inhibitory protein, κB (e.g. IκB-α, IκB-β, IκB-γ, IκB-ε), which resides in the cytoplasm. Specific (TNF-α, IL-1β), as well as unspecific (oxidative stress, UV radiation) signals can activate the NF-κB pathway (Figure), starting with the dissociation of the inhibitor from the NF-κB-complex and

the entrance of NF-κB into the nucleus. Here, the NF-κB complex can bind to DNA and activate the transcription of various target genes [56], many of which are inflammatory and immunoregulatory [57]. Blocking NF-κB activation, anywhere in the cascade, will lead to a repression of the transcription of the target genes and thus reduced inflammation.

Figure:

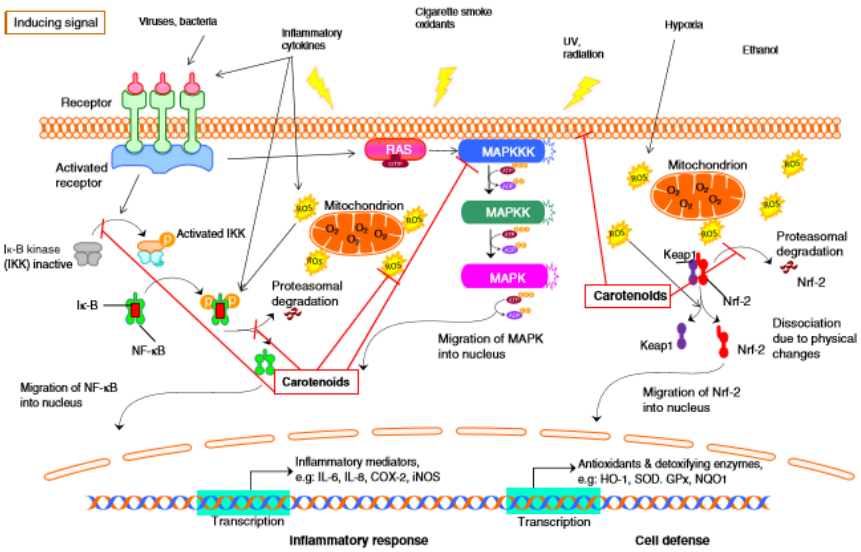

Figure. Inflammatory signaling pathways and carotenoids. Under resting conditions, inactive NF-κB is bound to its inhibitory protein IκB in the cytoplasm. After stimulation with e.g. oxidative stress, inflammatory cytokines, or hypoxia, IκB protein is phosphorylated by the IKK complex leading to the ubiquitination and proteasomal degradation of the IκB protein. This releases NF-κB, which can then translocate to the nucleus and the transcription of inflammatory cytokines can start [213]. It could be hypothesized that carotenoids or their derivatives can interact with cysteine residues of the IKK and/or NF-κB subunits and, as such, inactivate the NF-κB pathway [93]. Nrf2 is kept inactive by Keap1 in the cytosol by poly-ubiquitination and rapid degradation through the proteasome. During redox imbalance, the Keap1-Nrf2 association is disrupted and Nrf2 can dissociate from Keap1, entering the nucleus, and leading to the transcription of antioxidant and detoxifying enzymes, which promotes cytoprotection [58]. Carotenoids and their derivatives seem to interact with Keap1 by changing its physical properties [214]. Mitogen-activated protein kinases are a family of Ser/Thr protein kinases. MAPK signaling cascades are organized hierarchically into a 3-kinase architecture. MAPKs are phosphorylated and activated by MAPK-kinases (MAPKKs), which in turn are phosphorylated and activated by MAPKK-kinases (MAPKKKs). The MAPKKKs are in turn activated by interaction with the family of small GTPases and/or other protein kinases, connecting the MAPK module to cell surface receptors or external stimuli [215]. It is not known how carotenoids interact with the MAPK pathway. Part of the images from Motifolio drawing toolkits (www.motifolio.com) were used, with permission, in the figure preparation.

The Keap1-Nrf2 pathway (Figure) plays an important role in the cellular defense against endogenous or exogenous stress caused by ROS [58]. Under normal conditions, Nrf2 is bound to its repressor protein Keap1 in the cytosol, which leads to its degradation by ubiquitinylation [59]. Keap1 is a cysteine-rich protein with 27 cysteine residues, and its conformation can be modified by different oxidants and electrophiles, thereby leading to the liberation of Nrf2 and its translocation to the nucleus [58]. Modifications of the thiol residues of Keap1 lead either to a disrupted interaction with Nrf2, which can no longer be ubiquitinlyated, or to a dissociation of Nrf2 from Keap1. Either way, Keap1 is inactivated, and the newly synthetized Nrf2 can then translocate to the nucleus. It binds to the antioxidant response element (ARE), which leads to the expression of antioxidant and cytoprotective enzymes, for example, heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate cysteine ligase (GCL), or glutathione-S-transferases (GSTs) [59].

Mitogen-activated protein kinases are serine/threonine kinases. Their functions and regulations have been conserved from unicellular organisms to multicellular organisms. Mammalians have 3 well-characterized MAPK cascades (Figure): extracellular signal-regulated kinases (ERK1/ERK2), c-Jun N-terminal kinases (JNK1, JNK2, and JNK3), and p38 kinases [60]. Mitogen-activated protein kinases are typically organized in a “3-kinase architecture” that is activated by various extracellular stimuli, including IL-1β, TNF-α, lipopolysaccharide (LPS), and oxidative stress [61]. The 3-kinase architecture is composed of MAPK, a MAPK activator (MEK,

MKK, MAPK kinase), and a MEK activator (MEK kinase or MAPK kinase kinase). As they form a kinase cascade, each downstream kinase serves as a substrate for the upstream activator [62].

3.3 In vitro studies

3.3.1 General considerations and implications of ROS

Many *in vitro* studies (Table 1), especially those that include cellular models, have aided in establishing a link between carotenoids, oxidative stress, and inflammation. There are several advantages in employing cellular models to investigate the effects of carotenoids on inflammation-related pathways. These include the ability to investigate, under well-defined conditions, specified carotenoid concentrations and specific types of cells and therefore allowing for a large number of investigations that are suitable for hypothesis building and studying mechanistic effects. Most studies have investigated the more abundant carotenoids (eg, β-carotene, lutein, and lycopene), and occasionally, less frequently ingested ones, such as astaxanthin, are examined. The limitations of these cellular studies include the difficulty to conduct long-term studies due to limited cell-life, the missing interactions with other cells present *in vivo*, and the way of administration, that is, using carotenoids in solvents (eg, tetrahydrofuran and dimethylsulfoxide) rather than in lipoproteins or mixed micelles. This may also have percussions on their stability and cellular uptake, which normally occurs either passively or via transporters (SRBI, CD36, NPC1L1). In addition, many studies have used non-physiological high

concentrations, that is, greater than ca. 2 µM [25] (plasma and tissues) and ca. 20 µM (gut) [63]. Another controversy exists regarding the way in which inflammation is stimulated. Tumor necrosis factor-α [64], LPSs [65–67], IL-1β [68,69], H_2O_2[70,71], and bacterial [72,73] or viral stimulation [74,75] are among the most common stimuli *in vivo*. Typically, several of these factors are involved, activating multiple rather than individual pathways of inflammation [76].

Several cellular models for studying potential anti-inflammatory effects of carotenoids have been established. As carotenoid concentration is highest in the gut, several studies have focused on gastrointestinal epithelial cells such as Caco-2 [77], HT-29 cells [78], and human gastric epithelial adenocarcinoma (AGS) [79]. Because of their implication in many inflammatory mechanisms, monocytes/macrophages are another interesting target of carotenoids [80]. Carotenoids are mainly stored in adipose tissue and adipocytes (3T3-L1) [81,82], and keratinocytes (human primary keratinocyte [HPK]) [83] have been used in several studies. Retinal pigment epithelial cells (ARPE-19) may also accumulate in the skin and in the retina.

Reactive oxygen species could cause inflammation, and several *in vitro* studies have shown that β-carotene, lycopene, and lutein were able to reduce ROS production [84]. Lycopene has been suggested as a potent compound to decrease ROS, such as that generated by smoke, and to modulate redox-sensitive cell targets which include protein tyrosine phosphatases, protein kinases, MAPKs, and transcription factors [85]. For instance, lycopene at 2 µM was able

to reduce the effect of smoke on molecular pathways involved in inflammation, cell proliferation, and apoptosis as well as on carcinogen-activating enzymes, and it also inhibited the formation of smoke-induced DNA adducts and smoke-stimulated insulin-like growth factor signaling [85]. In another study, 2 μM of lycopene significantly reduced ROS levels by 60% in human monocytes (THP-1) when stimulated with cigarette smoke [86]. Lutein (20 μM) and β-carotene (20 μM) showed a significant reduction (20% and 10%, respectively) of ROS in AGS cells after stimulation with H_2O_2 [87]. Similarly, β-carotene (10 and 20 μM) significantly reduced ROS in AGS cells following Helicobacter pylori stimulation [88].

Reactive oxygen species can also be produced by poor coupling of the P450 catalytic cycle [89]. Several carotenoids, especially lycopene, have been shown to modulate liver metabolizing enzymes, such as cytochrome P4502 E1, thus resulting in anticancerous activity. In one example, lycopene reduced ROS production through the interaction with NAD(P)H oxidase and NOX-4 (a homologue of NAD(P)H) [90].

Table 1: Effects of carotenoids on different inflammatory mediators investigated in different cell cultures:

Carotenoids investigated	Cell type	Inflammatory stimulus	Carotenoid concentration	Inflammatory effects*	Reference
Astaxanthin	HPKs	UVB irradiation (80 mJ/cm^2)	8 µM	**NF-κB associated:** Reduced gene expression of COX-2 and IL-8, decreased PGE-2 and IL-8 secretion. No effect on NF-κB translocation to the nucleus. **MAPK associated:** Decrease of p38 and ERK phosphorylation, no effect on JNK phosphorylation.	Terazawa *et al.* [83]
Astaxanthin	U397 (human macrophage like cells)	H_2O_2 (100 µM)	10 µM	**NF-κB associated:** Decrease of IL-1β, Il-6 secretion. Blockage of NF-κB nuclear translocation.	Speranza *et al.* [94]
Astaxanthin	ARPE-19 (retinal pigment epithelia cells)	H_2O_2 (200 µM)	5-20 µM	**ROS:** Reduced level of ROS. **Nrf-2 associated:** Increased mRNA expression of NQO1, HO-1,GCLM and GCLC. Increased nuclear localization of Nrf-2.	Li *et al.* [105]
Astaxanthin	PC-12 (pheochromocytoma cell line)	MPP (N-methyl-4-phenylpyridinium) (500 µM)	10 µM	**ROS:** Reduced level of ROS by 22%. **Nrf-2 associated:** Decreased mRNA expression of NOX-2 by 24%. Increased mRNA expression of HO-1 by 117%. Increased Nrf-2 protein by 100%.	Ye *et al.* [107]

Table 1: continued

Carotenoids investigated	Cell type	Inflammatory stimulus	Carotenoid concentration	Inflammatory effects*	Reference
β-carotene	AGS (human gastric epithelial cells)	H. pylori (bacterium/cell ratio 300:1)	10 and 20 µM	**ROS:** Reduced level of ROS. **NF-κB associated:** 90% decrease of iNOS and COX-2 mRNA expression and NO and PGE-2 protein levels. Dose-dependent decreased DNA binding activity of NF-κB, via prevention of IκB degradation. **MAPK associated:** Inhibition of p38, ERK, JNK phosphorylation.	Jang *et al.* [88]
3-hydroxy-β-damascone	HT-29 (human colon adenocarcinoma cells)	n.i.	20 and 50 µM	**Nrf-2 associated:** Induction of NQO1. Increased Nrf-2 transcriptional activity.	Gerhäuser *et al.* [100]
RA	SH-SY5Y (human neuro-blastoma cell line)	n.i.	10 µM	**Nrf-2 associated:** Increased mRNA expression of Nrf-2 and NQO1.	Zhao *et al.* [101]
ATRA	SK-N-SH (human neuroblastoma cell line)	n.i	10 µM	**NF-κB associated:** Degraded IκB-β and increased NF-κB DNA binding activity.	Kiningham *et al.* [206]
RA	MCF-7 (human mammary cancer cells)	n.i.	1 µM	**Nrf-2 associated:** Decreased binding activity of Nrf-2 to ARE.	Wang *et al.* [103]
β-carotene or Lutein	AGS (human gastric epithelial cells)	H_2O_2 (100 µM)	20 µM	**ROS:** 10% reduction of ROS by β-carotene and 20 % reduction by lutein. **NF-κB associated:** Reduced IL-8 mRNA expression and protein levels. Lutein 10% higher effect. Decreased DNA-binding activity of NF-κB.	Kim *et al.* [87]
β-carotene or astaxanthin or capsanthin or bixin	K562 (human myelogenous leukemia cells)	n.i.	5-50 µM	**Nrf-2 associated:** Upregulation of Nrf-2 expression.	Zhang *et al.* [98]

Table 1: continued

Carotenoids investigated	Cell type	Inflammatory stimulus	Carotenoid concentration	Inflammatory effects*	Reference
Lutein or Zeaxanthin	ARPE-19 (human retinal pigment epithelial cells)	A2E (lipofuscin fluorophore) (10 µM)	10 µM	**NF-κB associated:** Decrease of IL-8 mRNA expression and protein levels.	Bian *et al.* [207]
Lycopene	RAW264.7 (murine macrophages)	LPS (1 ng/ml)	0.5; 1 , 2 µM	**NF-κB associated:** 30-40% reduction of IL-6 and IL-1β mRNA expression. **MAPK associated:** Decrease of JNK phosphorylation (no effect on p38 and ERK1/2 phosphorylation).	Marcotorchino *et al.* [82]
Lycopene	THP-1 (human monocytic cell line)	Cigarette smoke (0.5%)	2 µM	**ROS:** Reduced ROS production. **NF-κB associated:** Decrease of IL-8 mRNA expression and protein levels. Decrease of NF-κB DNA binding activity.	Simone *et al.* [86]
Lycopene	RAW264.7 (murine macrophages)	LPS (1µg/ml)	1-10 µM	**NF-κB associated:** Decreased mRNA of NO and IL-6. Inhibition of NO and IL-6. Inhibition of IκB and phosphorylation and degradation and NF-κB translocation. **MAPK associated:** Decrease of p38 and ERK phosphorylation but no effect on JNK phosphorylation.	Feng *et al.* [111]
Lycopene	THP-1 (human monocytic cell line)	7-ketocholesterol (25 µM)	2 µM	**ROS:** Reduced ROS production through reduction of NOX-4 and NAD(P)H oxidas **MAPK associated:** Decrease of p38, ERK and JNK phosphorylation.	Palozza *et al.* [110]
Lycopene	3T3-L1 (murine preadipocytes)	TNF-α (15 ng/ml)	2 µM	**NF-κB associated:** Decreased mRNA of IL-6 by 40 %. Inhibition of IKKα/β phosphorylation.	Gouranton *et al.* [81]

Table 1: continued

Carotenoids investigated	Cell type	Inflammatory stimulus	Carotenoid concentration	Inflammatory effects*	Reference
Fucoxanthin	BNL CL.2 (mouse hepatic cells)	n.i.	0.5-5 µM	**ROS:** Significant increase of ROS. **Nrf-2 associated:** Increase of nuclear Nrf-2 protein, increased binding of Nrf-2 to ARE. Increased mRNA of HO-1, NQO1. **MAPK associated:** Increased phosphorylation of ERK/p38.	Liu *et al.* [104]
Lycopene or β-carotene or phytoene or astaxanthin	HepG2 (human hepatocellular carcinoma cells) and MCF-7	n.i.	50 µM	**Nrf-2 associated:** Increase of Nrf-2-translocation to the nucleus in HepG2 cells. Activation of ARE by 3 to 4 fold in MCF-7 and HepG2 cells (lycopene concentration 6 µM)	Ben-Dor *et al.* [51]

Abbreviations: n.i. = no information; NO= nitric oxide; GCLM= glutamate cysteine ligase modulatory subunit; GCLC= glutamate cysteine ligase catalytic subunit; NOX-2/4= NAD(P)H oxidase; NADPH= nicotinamide adenine dinucleotide phosphate.
*All effects were significant.

3.3.2 Inflammation factors associated with NF-κB

Several studies have shown that carotenoids can reduce NF-κB activation [52,86,91,92], although it could be hypothesized that not (only) the intact carotenoids but also their derivatives are the key players in NF-κB regulation. Carotenoid derivatives, such as apocarotenals, contain electrophilic groups that can interact with the cysteine residues of IκB kinase (IKK) and NF-κB subunits (p65), thereby inactivating the NF-κB pathway [93].

Whichever the causal agent, it has been shown that β-carotene at 2 to 20 μM reduced the expression of several downstream targets of NF-κB, including iNOS and COX-2, by 90% in bacterially infected AGS cells [88]. In the same study, β-carotene also decreased the secretion of nitric oxide (NO) and PGE-2. Lycopene, at much lower concentrations (0.5-2 μM), showed similar effects on inflammatory cytokines and signaling pathways, by reducing IL-6 and Il-1β secretion by 30% to 40% in LPS-stimulated RAW264.7 cells [82]. Lycopene (2 μM) decreased the IL-6 messenger RNA (mRNA) expression in preadipocytes (3T3-L1) by 40% and in mature adipocytes (3T3-L1) by 37% [81], suggesting that it may play an important role in the homeostasis of adipose tissue. At 2 M, lycopene reduced IL-8 mRNA expression and protein levels, as well as NF-κB DNA binding and the production of ROS in cigarette smoke–stimulated human monocytic (and macrophage-like) cells (THP-1) [86], therefore supporting that the idea that lycopene may be an interesting candidate for further testing *in vivo*, such as in smokers. Another research group analyzed the effect of astaxanthin on several inflammatory mediators and NF-κB in H_2O_2-stimulated human

macrophage-like cells (U397) and found that it lowered IL-1β and IL-6 secretion and blocked NF-κB translocation to the nucleus [94]. In addition to blocking translocation, studies demonstrated that β-carotene could inhibit H_2O_2 and *H. pylori* induced NF-κB–DNA binding in the nucleus of AGS [87,88] and macrophage cells [95] at concentrations of around 2 to 25 μM. This was accomplished via the prevention of the degradation of the inhibitor IκB [88] in the cytosol, thus suggesting further downstream general anti-inflammatory effects.

Some studies have investigated the relationship between carotenoids and UV-induced inflammation, because carotenoids may be distributed in the human skin [96,97]. Studies in UV-B irradiated HPKs revealed that astaxanthin at 8 μM reduced COX-2 and IL-8 gene expression and also PGE-2 and IL-8 protein secretion [83]. It was also tested whether astaxanthin influenced NF-κB translocation, but no effect was seen, perhaps due to transient effects of the latter.

3.3.3 Inflammation factors associated with Nrf2 and MAPK

Recently, due to the typical strong antioxidant effects of lycopene, the effects of lycopene on Nrf2 have been reviewed [90], and it was noted that lycopene was able to up-regulate the Nrf2 binding ARE system in several cell types such as MCF-7 breast cancer and HepG2 liver cells [51]. In a study by Zhang *et al.* [98], α-carotene, astaxanthin, capsanthin, and bixin at 5 to 50 μM up-regulated the expression of Nrf2 in K562 leukemia cells. In a study by Ben-Dor *et al.* [51] that focused on MCF-7 cells, expressions of reporter genes fused with ARE

sequences after various carotenoid treatments (lycopene, phytoene, a-carotene, and astaxanthin) at 2 to 10 µM were compared. Their results showed that lycopene had the strongest effect and, hence, suggested that it is a good activator for the nuclear translocation of Nrf2. This may seem somewhat surprising due to its low cytosolic solubility and apolarity, which constitutes a poor activator of Keap1, and it may shed further doubts on whether lycopene, rather than its apocarotenal metabolites, are the bioactive constituents. Because carotenoids such as lycopene lack an electrophilic group, which appears to be required to react with Keap1, it is suggested that not the parental carotenoids, but metabolites following BCO1 and BCDO2 cleavage are the responsible bioactives [99]. An example includes the 10,10′-apocarotenal derivative of lycopene, which is shown to be active in MCF-7 and prostate adenocarcinoma (LNCaP) cells. In general, apocarotenals showed higher activity than apocarotenoid acids in terms of EpRE/ARE transactivation. In another study, 3-hydroxy-a-damascone, a carotenoid derived polar flavor (apocarotenoid), induced NQO1 in HT-29 cells after 10-, 20-, or 50-µM exposure [100], with the latter 2 concentrations being significant. It also appears that retinoic acid (RA), after cleavage of α-carotene by BCO1, is effective in enhancing Nrf2 translocation. Similarly, in a study on neuroblastoma cells (SH-SY5Y), RA at 5 to 40 µM induced mRNA expression of Nrf2 and NQO1, dose dependently [101]. Thus, all these studies are supportive of the idea that more polar carotenoid metabolites can activate Nrf2 translocation.

It also appears that oxidative stress, caused by rather high concentrations (>10 µM) of RA, stimulates Nrf2 translocation [102], whereas lower concentrations (0.1-1.0 µM) may inhibit Nrf2

translocation, indicating a biphasic activity behavior [102,103]. However, this is not confirmed in all studies. For example, in the study by Ben-Dor *et al.* [51], ARE activation did not depend on the level of intracellular ROS or reduced glutathione, thus suggesting that this activation was not related to antioxidant activity. At comparable low concentrations, fucoxanthin (above 0.5 µM) in hepatic cells (BNL CL.2) increased nuclear Nrf2 protein accumulation, and levels of mRNA and protein expression of downstream HO-1 and NQO1 [104]. Of note, fucoxanthin also increased ROS levels and phosphorylated ERK and p38 intracellularly indicating that prooxidant properties could result in beneficial effects.

In a recent study by Li *et al.* [105], astaxanthin protected retinal epithelial cells (ARPE-19) from H_2O_2 induced oxidative stress by inducing nuclear localization of Nrf2, reducing intracellular ROS, and up-regulating NQO1, HO-1, glutamate cysteine ligase modulatory subunit, and glutamate cysteine ligase catalytic subunit mRNA expression, although astaxanthin concentrations were comparatively high at 5 to 20 µM. In the same study, the involvement of P-Act and its downstream target Nrf2 was further suggested by inhibitors of P-Act, diminishing the positive effects of astaxanthin in ARPE-19 cells. The influence on retinal cells has been met with increasing interest due to the relation of age-related macular degeneration (AMD) and retinal carotenoid concentrations [106]. As carotenoids are also able to cross the blood-brain barrier and are found in brain cells, several cellular models of neuroprotection exist, such as for Parkinson's disease. In a recent study on neuronal PC-12 cells [107], astaxanthin protected against N-methyl-4-phenylpyridinium induced oxidative stress at 10

μM, reduced intracellular ROS and NOX2, and increased Nrf2 and HO-1 protein expression; however, realistic concentrations in this tissue are expected to be lower (up to 0.5 μM) [108].

Only a few studies have investigated the effect of carotenoid cellular exposure on targets of the MAPK pathway, although interactions have been shown for lutein, zeaxanthin, and lycopene at the MEK activator level in the 3 different MAPK cascades [109]. It has been demonstrated that astaxanthin (4 or 8 μM) decreased the phosphorylation of p38 and ERK, but no effect was found on JNK in human keratinocytes [83]. Similar effects were seen for α-carotene, preventing the phosphorylation of ERK, JNK, and p38 [88] in the cytosol. In a study by Palozza *et al.* [110], lycopene suppressed MAPK phosphorylation in oxysterol stimulated macrophages and prostate cancer cells. In another study, lycopene decreased JNK phosphorylation but showed no effect on p38 and ERK phosphorylation in LPS-stimulated RAW264.7 cells [82]. This finding contrasts with an investigation where lycopene blocked p38 and ERK phosphorylation but did not affect JNK phosphorylation in RAW264.7 cells [111]. A possible explanation might be the slightly different concentrations of lycopene, which ranged from 0.5 to 2 μM in one [82] and 1 to 10 μM in the other study [111]. Also, the time of exposure (often varying between 6 and 48 hours) and the cell passage number could have affected the results.

Table 2. Effects of carotenoids on inflammation in animal models

Animals involved	Carotenoid investigated	Concentration of carotenoid	Stimuli	Treatment length	Tissue/cells sampled	Main effects	Reference
Rats	Annatto extract or β-carotene	0.1 % in the diet	n.i.	7 d	Neutrophils	**ROS:** Reduced ROS production. Increased mRNA expression of SOD CAT, p22, p47.	Rossoni Junior *et al.* [114]
Rats	Lycopene	10 mg/kg bw.	Sodium fluoride	5 wks	Red blood cells, heart and brain	**ROS:** Reduced MDA, total nitrate/nitrite and glutathione.	Mansour *et al.* [117]
Rats	Lycopene	40 mg/kg bw.	Mercury chloride	Single doses	Renal tissues	**ROS:** Decreased ROS levels by 16%. Decreased MDA and GSH. Increased GPx and SOD.	Yang *et al.* [118]
Rats	Astaxanthin	20 mg/kg bw.	Alloxan	30 d	Neutrophils	**ROS:** Reduced TBARS and H_2O_2 levels.	Marin *et al.* [119]
Rats	Lycopene	1-4 mg/kg bw.	Streptozotocin	10 wks	Serum	**NF-κB associated:** Reduced TNF-α production.	Kuhad *et al.* [130]
Rats	Lycopene	10, 15 and 20mg/kg bw.	n.i.	12 wks	Serum	**NF-κB associated:** Reduced VCAM-1, MCP-1, IL-8.	Liu *et al.* [131]
Rats	Lutein	1-100mg/kg bw.	Streptozotocin	Single injection	Aqueous humor	**NF-κB associated:** Inhibited NF-κB activation. Decrease of NO, PGE-2, IL-6, TNF- α, CCL2 and CXCL2.	Kijlstra *et al.* [134]
Rats	Fucoxanthin	0.1, 1 or 10 mg/kg bw.	LPS	Single injection	Aqueous humor	**NF-κB associated:** Reduced PGE-2, NO, TNF-α levels.	Shiratori *et al.* [136]
Rats	Lycopene	1.1 or 3.3 mg/kg bw.	Alcohol	11 wks	Plasma and liver	**NF-κB associated:** Induction of CYP2E1 protein and TNF-α mRNA.	Veeramachaneni *et al.* [143]

Table 2: Continued

Animals involved	Carotenoid investigated	Concentration of carotenoid	Stimuli	Treatment length	Tissue/cells sampled	Main effects	Reference
Rats	Lycopene	6 mg/kg bw.	Cisplatin	Single injection	Kidney cells	**ROS:** Increased levels of SOD, GPx and CAT. **NF-κB associated:** Decrease of NF-κB expression. **Nrf-2 associated:** Increased Nrf-2 and HO-1 expression.	Sahin *et al.* [145]
Rats	Astaxanthin	25 mg/kg bw.	Cyclophosphamide	10 d	Liver	**Nrf-2 associated:** Increase of NQO-1 and HO-1.	Tripathi *et al.* [146]
Rats	Crocin	10 mg/kg bw.	Cyclophosphamide	6 d	Liver and serum	**ROS:** Increase in SOD, CAT, GSTs, GPx.	Jnaneshwari *et al.* [138]
Rats	Crocin	10 or 20 mg/kg bw.	Freund's complete adjuvant	14 d	Serum, liver and Spleen tissue	**NF-κB associated:** Decreased IL-1β, IL-6, TNF-α and COX-2 and PGE-2 levels. **ROS:** ROS decrease by 98 %. Reverted GSH levels.	Hemshekhar *et al.* [139]
Rats	Crocetin	50 mg/kg bw.	hemorrhagic shock	Single administration	Renal blood	**NF-κB associated:** Decreased NO, TNF-α and IL-6.	Wang *et al.* [140]
Rats	β-carotene	0.05 % in diet	azoxymethane	33 wks	Colonic mucosa	**NF-κB associated:** Slight reduction of COX-2 expression.	Choi *et al.* [141]
Mice	β-carotene or Lycopene	0.5g/kg bw.	Cigarette smoke	4 wks	Lung	**ROS:** regulated expression of Cytochrome P450.	Aung *et al.* [126]
Mice	β-carotene	0.6 % in diet	n.i.	10, 15 or 20 wks	Liver	**NF-κB associated:** Reduced mRNA of VCAM-1, Il-1α, MCP-1, IFN-γ.	Harari *et al.* [127]

Table 2: Continued

Animals involved	Carotenoid investigated	Concentration of carotenoid	Stimuli	Treatment length	Tissue/cells sampled	Main effects	Reference
Mice	β-carotene	150 mg/kg bw.	n.i.	14 wks	Lung, liver and white adipose tissue	**NF-κB associated:** Decreased expression of genes involved in interferon production/regulation.	Van Helden *et al.* [128]
Mice	RA	0.5 mg/kg bw.	n.i.	9 wks	CD4(+) cells and arthritic joints	**NF-κB associated:** Decreased IL-17, IL-6, IL-1β and iNOS expression. Downregulated NF-κB.	Kwok *et al.* [92]
Mice	Lycogen	1 mg/kg bw.	Dextran sodium sulfate	6 d	Plasma	**NF-κB associated:** Reduced expression of TNF-α and IL-1β.	Liu *et al.* [129]
Mice	Astaxanthin	1, 10 or 100 mg/kg bw.	n.i.	3 d, injections	Retinal pigment epithelium-choroid tissue	**NF-κB associated:** Reduced IL-6, VEGF, MCP-1 and ICAM-1. Suppression of NF-κB activation, translocation and IκB degradation.	Izumi-Nagai *et al.* [137]
Mice	Lutein	1, 10 and 100 mg/kg	LPS	Single administration	Aqueous humor and iris-ciliary body	**NF-κB associated:** Dose dependent reduction of NO, TNF-α, IL-6, PGE-2 concentration; NF-κB activation.	Jin *et al.* [135]
Mice	Lutein	0.1 % in diet	streptozotocin	1 month	Retina	**ROS:** Reduced ROS production. **MAPK associated:** Inhibit ERK activation.	Sasaki *et al.* [120]
Mice	Astaxanthin	100mg/kg bw.	H_2O_2	4 times	Retina	**ROS:** Reduced retinal damage and oxidative DNA damage.	Nakajima *et al.* [121]
Mice	Vitamin A and RA	10:1 molar ratio	Hyperoxia	7 or 14 d	Lung	**Nrf-2 associated:** Decreased DNA damage. Reduction of Nrf-2 proteins.Decrease of INF-γ and macrophage inflammatory protein 2-α mRNA.	James *et al.* [147]
Mice	RA	0.5 mg/mouse	n.i.	n.i.	T cells	**Other pathways:** Suppression of IFN-γ producing CD4(+) and CD8(+) T cells. Suppression of STAT4 expression.	Van *et al.* [152]

Table 2: Continued

Animals involved	Carotenoid investigated	Concentration of carotenoid	Stimuli	Treatment length	Tissue/cells sampled	Main effects	Reference
Mice	RA	400 µg/mouse	ovalbumin	Single administration	Lymphocytes	**Other pathways:** Inhibition of Th-2 cells and Th17 related cytokines.	Wu *et al.* [208]
Mice	Lycopene	8 or 16 mg/kg bw.	ovalbumin	3 d	Lung	**Other pathways:** Decreased IL-4 mRNA. Increased IFN-γ and T-bet mRNA.	Lee *et al.* [156]
Mice	Vitamin A	250 IU/g diet	ovalbumin	3 wks	Lung	**Other pathways:** Decreased Il-4 and IL-5 release.	Schuster *et al.* [209]
Mice	Lycopene	100 mg/kg bw.	n.i.	Single dose	Different organs	**Other pathways:** Induction of RARE-mediated cell signaling.	Aydemir *et al.* [153]
Mice	Lycopene	0-20 µM	LPS	Single administration	Endothelial cells	**Other pathways:** Inhibition of HMGB1 release and HMGB1-mediated TNF-secretory phospholipase A2-IIA and HMGB1- mediated pro-inflammatory responses.	Lee *et al.* [210]
Dogs	Astaxanthin	20 mg/d	n.i.	16 wks	Leukocytes	**ROS:** Improved mitochondrial function and plasma GPx and NO levels.	Park *et al.* [122]
Chicken	Astaxanthin	100 ppm	LPS	2 wks	Liver and spleen	**NF-κB associated:** Increase in iNOS, IL-1, IL-6 and IFN-γ mRNA expression.	Takahashi *et al.* [142]

Abbreviations: n.i. = not investigated; CCL2= chemokine (C-C motif) ligand 2; CXCL2= chemokine (C-X-C motif) ligand 2; IFN-γ= interferon gamma; VEGF= vascular endothelial growth factor; RARE, RA response element; TBARS, thiobarbituric acid reactive substance.

3.3.4 Summary and outlook

Despite the limitations of cellular studies to model complex *in vivo* environments, in conjunction with the often high concentrations of carotenoids that are investigated, several carotenoids, at concentrations that are physiologically plausible, have indicated that they are able to reduce ROS. Because of the relation of ROS and inflammation, it is not too surprising that carotenoids were also found to positively modulate markers of inflammation and oxidative stress, especially those related to the NF-κB or Nrf2 pathway, respectively. This occurred by blocking NF-κB translocation to the nucleus and removal of the Nrf2 repressor Keap1. The strength of the effect across various carotenoids appears difficult to predict, as studies often differ in many parameters, which include cell type, incubation time, and concentration, and the effects may not strictly be related to intracellular oxidative stress. An important factor deserving further investigation is the potential carotenoid metabolites which, also depending on the cell type and the ability to cleave carotenoids via eg, BCO1 or BCDO2 into apocarotenals, appear to be in part responsible for the observed anti-inflammatory reactions. This is plausible given that free carotenoid concentration in the cytosol (where Nrf2 and NF-κB reside) are typically low and require binding to cysteine residues of these transcription factors, therefore favoring polar compounds. Also, carotenoid concentration in such studies is likely to influence the results. Although Nrf2 translocation appears to be more prominent at rather higher concentrations (>1 μM), possibly via increasing oxidative stress due to prooxidant effects of carotenoids or their

metabolites, lower concentrations may have rather inhibitive effects on Nrf2.

3.4 Animal studies

3.4.1 General aspects and implications of ROS

Compared with cellular trials, animal models (Table 2) allow for studying the effects under more complex, that is, realistic, physiological conditions. Compared with human studies, they are easier to coordinate under more standardized conditions and allow for a higher accessibility to tissues. As a drawback, many animals metabolize carotenoids differently from humans [112]; their cleavage rate by BCO1 and BCDO2, such as in mice (being much higher), can be quite altered compared with humans. In addition, in many animal models, carotenoids were administered at supraphysiological doses that were comparable to ca. 6 mg/kg body weight (bw) when setting 0.5 mg/kg bw in humans as a limit for a physiological dose, applying the human equivalent dose formula for comparing doses across species [113]. Animal models resembling the human carotenoid metabolism include Mongolian gerbils and various types of monkeys [112].

By providing foods rich in carotenoids, several studies have aimed at reducing ROS and their negative impact on inflammation. For example, annatto extract or β-carotene added at 0.1% to the diet of rats for 7 days decreased ROS production in neutrophils and increased mRNA concentrations of superoxide dismutase (SOD), catalase (CAT), p22 (phox), and p47 (phox), which are components of the

electron transfer elements of nicotinamide adenine dinucleotide phosphate oxidase [114].

However, most studies investigated isolated carotenoids that were added to the diet. Administration of lycopene in isolated form has received some attention because of its strong antioxidant effects *in vitro* [115]. Similar to cellular studies, lycopene ameliorated the negative effects of ROS produced during anticancer chemotherapy [116]. In another study on rats, daily lycopene administration of 10 mg/kg bw for 5 weeks normalized oxidative stress (caused by sodium fluoride exposure), assessed as plasma MDA and total nitrate/ nitrite [117]. Lycopene also ameliorated oxidative stress in tissues, for example, in renal tissues (glutathione [GSH], MDA, SOD), when administered as a single dose (40 mg/kg bw) prior to mercury poisoning [118].

Astaxanthin (20 mg/kg), administered to diabetic rats for 30 days, reduced oxidative stress (measured by thiobarbituric acid reactive substance, H_2O_2) in neutrophils, although it did not go back to basal levels [119]. In another diabetes model, lutein (0.1% in the diet) in rats prevented ROS-related retina degeneration after 4 months of supplementation [120]. Positive effects on the retina were also found for astaxanthin in mice, but they received very high doses, that is 4 times 100 mg/kg [121]. These results support the positive effects of carotenoids for eye health. In larger animals, for example, dogs, 20 mg/d of astaxanthin for 16 weeks improved mitochondrial function and markers of oxidative stress, including plasma glutathione peroxidase (GPx) and NO [122]. These observations were attributed, in addition to indirect effects on intracellular signaling (especially Nrf2) to the

following: (a) quenching of singlet oxygen, (b) reaction with free radicals, (c) acting as an antioxidant via reactivating vitamin E or vitamin C, or (d) reducing DNA damage [123]. Further additional modes of action may exist. Several animal studies have suggested that carotenoids can up-regulate the P450 cytochrome mono-oxygenase family and may come into play when detoxifying xenobiotic compounds. For instance, several enzymes such as P450E1 in rat liver [124] or CYP 1A1/2 2B1/2 and 3A [125] were up-regulated in a dose-dependent manner by lycopene, and both lycopene and β-carotene activated the P450 A1 gene in mice [126].

3.4.2 Implication of the NF-κB pathway

In cellular studies, NF-κB or downstream cytokines have been among the most studied markers of inflammation in animal studies after carotenoid exposure. Positive effects on inflammatory genes, such as those implicated in the expression of IL-1α, INF-γ, monocyte chemotactic protein 1 (MCP-1), and vascular cell adhesion protein 1 (VCAM-1), were found after feeding a diet of 0.6% β-carotene (50% in 9-cis form) to rats (in which atherosclerotic lesions were induced by a high-fat diet) for 11 weeks [127]. In another study with BCO1-deficient mice, 14-day β-carotene supplementation (150 mg/kg in diet) reduced mRNA expression of proinflammatory genes involved in interferon production/regulation in the lung, liver, and white adipose tissue [128]. This suggests that effects were independent from vitamin A active compounds, although BCDO2 may still have been active. As with cellular models, metabolites of carotenoids have been shown to be effective in altering the inflammatory processes. In arthritis mice models [92], RA intraperitoneal (IP) at 0.5 mg/kg for 9 weeks reduced

several inflammatory parameters, including IL-17 in CD4(+) T cells and serum IgG/IgG2, down-regulated NF-κB in CD4(+) T cells, and reduced iNOS, IL-6, and IL-1β in tissues.

Carotenoids other than β-carotene also received some attention. In a rodent study, colitis induced in mice by dextran–sodium sulfate was reduced by the administration of lycogen (at 1 mg/kg for 6 days), which is a trademark extract rich in carotenoids. Both TNF-α and IL-1β in plasma were also reduced [129]. In diabetic induced rats, lycopene given at 1 to 4 mg/kg for 10 weeks reduced inflammation, as measured by TNF-α in serum, and limited cognitive decline, as indicated by the Morris water maze test [130]. In a hyperhomocysteinemic rat model, lycopene at 10, 15, and 20 mg/kg taken for 12 weeks reduced serum markers of inflammation such as VCAM-1, MCP-1, and IL-8, thus indicating antiatherogenic effects [131].

Because AMD is related to inflammation processes [132], and lutein and zeaxanthin have been especially related to AMD [133], many animal models focusing on the retina have investigated the potential benefits of carotenoids. As reviewed by Kijlstra [134], lutein intravenous injections (doses between 1 and 100 mg/kg) reduced aqueous humor NO, PGE-2, IL-6, TNF-α, various chemokines, and NF-κB activation in the iris ciliary body in a rat model, when administered prior to endotoxin induced uveitis [135]. In a similar rat model of LPS-induced uveitis, fucoxanthin injected intravenously at 0.1, 1.0, or 10 mg/kg after LPS administration reduced PGE-2, NO, and TNF-α concentration in the aqueous humor [136]. In a mouse model of AMD, astaxanthin given ip to mice (10 or 100 mg/kg bw) reduced NF-κB activation and inflammation-related molecules such as vascular endothelial growth factor, IL-6, ICAM-1, and MCP-1 in

retinal pigment epithelium tissues [137]. Taken together, these results suggest that inflammatory processes in the retina involved in AMD may be altered by considerable doses of carotenoids. A further hint toward the bioactivity of polar carotenoids and their metabolites comes from studies with crocin. This natural, more polar carotenoid attenuated cyclophosphamide induced hepatotoxicity in rats after a 6-day administration of 10 mg/kg. In addition to various markers of oxidative stress, inflammatory markers that included cytokines were significantly decreased [138]. In a rat model of arthritis (induced by *Mycobacterium tuberculosis*), 10 or 20mg/kg crocin for 14 days reduced plasma levels of inflammatory markers, including TNF-α, IL-1β, IL-6, COX-2, and PGE-2, as well as markers of ROS, such as GSH [139]. In rats, crocin administered at 50mg/kg after hemorrhagic shock–induced acute renal failure reduced negative effects by decreasing NO, TNF-α, and IL-6 and also attenuating NF-κB expression [140].

Some conflicting results exist and deserve to be mentioned because there is the possibility of severe underreporting of nonsignificant or even negative results. In a study by Choi *et al.* [141] β-carotene given at 0.5 % for 33 weeks to rats with induced colon carcinogenesis failed to reduce inflammatory markers such as COX-2. In a broiler study by Takahashi *et al.* [142] the addition of astaxanthin at 100 ppm to the diet showed no positive effect on inflammation, but it aggravated LPS-stimulated inflammation, which was measured by mRNA expression of iNOS, IL-1, IL-6, and IFN-γ in the liver. The reasons for these observations remain unknown, but altered metabolism of carotenoids in birds, low dosing of carotenoids, or overlayering effects

with the co-consumed corn (also containing carotenoids) could have played a role.

Even negative effects of carotenoids have been reported. When feeding physiological doses of 1.1 or 3.3 mg/kg bw (equivalent to ca. 45 mg/kg in a 70-kg human) for 11 weeks to rats receiving high doses of ethanol, the higher lycopene dose increased hepatic TNF-α mRNA, thus suggesting a prudent approach when administering carotenoids when chronic alcohol abuse is involved [143]. Although the reasons remain unknown, in the same study, it was suggested that alcohol slowed down BCDO2 enzymatic activity and the formation of lycopene metabolites. The resulting elevated tissue concentrations of the native carotenoid could have caused prooxidative effects [144].

3.4.3 Implication of the Nrf2 and other pathways

As the importance of Nrf2 in oxidative stress and inflammation related pathways has only recently been discovered, very few carotenoid studies investigating Nrf2 have been conducted in animals. As cancer chemotherapy may cause oxidative stress, a few studies have attempted to ameliorate this undesired side effect. In a study with cisplatin (used in chemotherapy), lycopene ameliorated nuclear Nrf2 and HO-1 decreases in rats receiving 6% lycopene and 1.5% other carotenoids at 6 mg/kg (phytoene, phytofluene) in their diet for 6 days (*i.e*. 360 μg/kg final lycopene) [145]. Expression of NF-κB p65 was also reduced, and the formation of downstream SOD, GPx, and CAT was up-regulated in kidney cells. In another rat study, astaxanthin increased NQO-1 and HO-1 expression in rats, after exposure to either cyclophosphamide with or without pretreatment of 25 mg/kg

astaxanthin [146]. In line with cellular studies, vitamin A active carotenoid metabolites (*i.e*. RA) improved hyperoxia induced depressed lung function in mice, reduced DNA damage, protein oxidation, interferon-γ, and macrophage inflammatory protein-2α mRNA, thus reducing Nrf2 proteins [147].

In addition to primary inflamed cells, it should not be overlooked that cytokines may also attract cells of the immune system, such as macrophages, which can then further stimulate inflammation responses. Carotenoids may also have effects on these attracted immune cells. It is well acknowledged that RA and other vitamin A active compounds are required for optimal functioning and maturation of the immune system [148], and studies have shown that at a later age, they can have pronounced effects on the immune system, such as on CD4(+) and CD8(+) T cells, for example, reducing their inflammatory potential [149,150]. However, it is possible that pathways other than NF-κB and Nrf2 also play a role, such as signal transducers and activators of transcription (STAT) [151]. For example, in a model of type 1 diabetes, all-trans retinol at 0.5 mg/mouse reduced STAT4 expression required for IFN-γ production and immune cell response [152]. These activities of RA are of interest because other carotenoid metabolites may also show similar behavior. In a mouse study by Aydemir et al [153], it was shown that lycopene can also activate the RA receptor (RAR) and the RA response element. Similar results were found by Harrison *et al.* [154]. This study demonstrated that various β-apocarotenoids were able to bind to RARα, RARβ, and RARγ receptors, thereby opening the door to many immune-related reactions of carotenoid metabolites.

Possibly due to similar activities, swelling and inflammation of the paw in mice (induced by carrageenan) receiving a pepper carotenoid extract (a mixture of carotenoids, *i.e*. β-carotene, violaxanthin, capsanthin, and capsorubin) were significantly reduced at 5, 20, or 80 mg/kg [155]. Such reduced involvement of inflammatory stimulating cells was also seen after lycopene administration. In a murine model of asthma (induced by ovalbumin), lycopene (8 or 16 mg injected IP for 3 days) reduced inflammation, as measured by infiltration of inflammatory cells (neutrophils, eosinophils, lymphocytes, and macrophages), into the peribrochilar and perivascular regions. It also decreased mRNA levels of IL-4 in bronchoalveolar lavage fluid but increased IFN-γ and T-bet (encoding for transcription factors responsible for development and also for IFN-γ production), thus suggesting an overall anti-inflammatory activity in upper airway diseases [156]. Lycopene administration (0-20 μM) also reduced permeability and migration of leukocytes to the peritoneal cavity in mice. This was explained by its influence on the high-mobility group box 1 (HMGB1), which is a nuclear protein responsible for the mediation of proinflammatory stimuli such as of multiple cytokines and chemoattraction of stem cells [157]. Further data on the effect of carotenoids on this protein are limited though.

Table 3. Effects of carotenoids or carotenoid rich food items on different inflammatory mediators and oxidative stress in humans.

Study type	Cell type or specimen	Test meals	Time frame	No. of subjects	Carotenoids investigated	Aspects studied	Main effects	Reference
Intervention studies with food items	Peripheral blood mononuclear cells	Fruits & vegetables (2 or 8 servings/d)	8 wks	64 men	n.i.	Fruit/vegetable consumption and immunological markers.	Consumption of fruits/ vegetables: no impact on TNF-α, IL-12 (2 and 8 servings/d) and C-reactive protein (2 servings/d). Consumption of 8 servings/d lowered C-reactive protein.	Watzl *et al.* [198]
	Urine and serum	Vegetables (2, 5 or 10 servings/d= 130 g, 237 g, 614 g)	3 wks	49 women	n.i.	Vegetable consumption and ox. stress, inflammation.	Consumption of vegetables: no effect on urinary 8-isoprostane F2α and serum C-reactive protein.	Crane *et al.* [199]
	Peripheral blood mono-nuclear cells	68 g avocado	1 consumption	11 individuals	n.i.	Avocado and inflammatory responses.	Avocado consumption reduced IL-6 secretion and NF-κB activation.	Li *et al*. [211]
	/	Fruits & vegetables	12 y	73286 women	α-and β-carotene, lycopene, lutein/zeaxanthin β-cryptoxanthin	Dietary carotenoids and CAD.	Significant inverse relation between intake of β-carotene and alpha-carotene rich foods and coronary artery disease.	Osganian *et al.* [212]
	/	Carotenoid rich foods (mostly carrot)	15 y	559 men	α-carotene β-carotene	Carotenoid rich foods and CVD.	α– and β-carotene rich food: lowered CVD mortality. Other carotenoids: no effect	Buijsse *et al.* [165]
	/	Tomato products	/	/	lycopene	Tomato and prostate cancer.	Tomato and tomato products might play a role in prostate cancer, when highly consumed.	Etminan *et al.* [166]
	/	Food rich in carotenoids	7-16 y	399765 individuals	α-and β-carotene, lycopene lutein/zeaxanthin β-cryptoxanthin	Carotenoid rich food and lung cancer.	Intake of β-cryptoxanthin rich food: inversely associated with lung cancer risk	Mannisto *et al.* [167]

Table 3: Continued

Study type	Cell type or specimen	Test meals	Time frame	No. of subjects	Carotenoids investigated	Aspects studied	Main effects	Reference
Intervention studies with food items	White blood cells	Tomatoes (360-728 g/d)	1 single dose	5 individuals	n.i.	DNA oxidation	Tomato consumption decreased levels of 8-hydroxyguanine	Rehman et al. [180]
	Peripheral lymphocytes	Tomato juice (330 ml/d 3-4 wks) Carrot juice (330 ml/d, 5-6 wks) Dried spinach in milk/water (10 g/d, 7-8 wks)	8 wks in total	23 men	lycopene β-carotene alpha-carotene lutein	DNA oxidation	Reduction of strand breaks in lymphocyte DNA. Reduction of oxidative base damage measured via COMET assay through the carrot juice.	Pool *et al.* [178]
	Urine	Tomato products	3 wks	12 women	lycopene	Tomato products and markers of lipid oxidation	Significant reduction of 8-iso-PGF2α due to tomato products consumption.	Visioli *et al.* [181]
	Leucocytes and prostate tissue	Tomato-sauce (30 mg lycopene/d)	3 wks	32 men	lycopene	Tomato sauce and prostate cancer.	Tomato sauce reduced oxidative DNA damage in leucocytes and in prostate tissue.	Chen *et al.* [182]
	Plasma	Tomato juice (500 ml/d)	4 wks	57 diabetic subjects	lycopene	Tomato juice and LDL oxidation.	Tomato juice consumption increased the resistance of LDL to oxidation.	Upritchard *et al.* [184]
	Skin	Tomato paste (55 g/d including 16 mg lycopene)	12 wks	20 women	lycopene	Tomato paste and cutaneous photodamage.	Protection against cutaneous photodamage through reducing mitochondrial DNA damage.	Rizwan *et al.* [186]
	Peripheral blood mononuclear cells	Tomato juice (330 ml/, 47.1 mg lycopene)	8 wks	53 individuals	lycopene	Tomato juice and cell mediated immunity.	Tomato juice decreased IL-2 and IL-4 secretion.	Watzl *et al.* [191]

Table 3: Continued

Study type	Cell type or specimen	Test meals	Time frame	No. of subjects	Carotenoids investigated	Aspects studied	Main effects	Reference
Intervention studies with food items	Whole blood	Tomato drink (5.7 mg lyco-pene, 3.7 mg phytoene, 2.7 mg phytofluene, 1 mg β-carotene, 1.7 mg α-tocopherol)	26 d	26 individuals	lycopene	Tomato based drink and markers of inflammation.	Tomato based drink decreased TNF-α levels.	Riso *et al.* [192]
	Serum	Tomato paste (200 g/d, 16 mg lycopene); pure lycopene (16 mg/d)	1 wk	30 men	lycopene	Tomato paste and lycopene and different target genes of prostate cancer cells.	Tomato paste: upregulated IGFBP-3 and Bax/Bcl-2 ratio and downregulated cyclin D1, p53 and Nrf-2. Lycopene con-sumption upregulated IGFBP-3, c-fos and μPAR.	Talvas *et al.* [200]
	Lymphocytes	Passata sauce (170 g pasta, ca. 7 mg/d lycopene)	3 wks	24 men	lycopene	Passata sauce on oxidative stress.	Consumption of passata sauce: no effect on HO- in lymphocytes.	Markovitch *et al.* [201]
Intervention studies with isolated carotenoids/ supplements	/	β-carotene (20 mg/d) & vitamin E (50 mg/d) suppl.	8 y	29133 smokers and non-smokers	β-carotene	B-carotene & vitamin E and lung cancer	Increased lung cancer risk in smokers	Albanes *et al.* [172]
	/	β-carotene (30 mg/d) & retinyl palmitate (25000 IU) suppl.	5 y	18314 smokers and non-smokers	β-carotene	β-carotene & vitamin A and lung cancer incidence	Increased lung cancer incidence in smokers	Omenn *et al.* [171]
	/	β-carotene (20mg/d) & vitamin E (50mg/d)suppl.	6 y	28519 men	β-carotene	Effect on stroke incidence in smokers	Increased risk of intracerebral hemorrhage	Leppälä *et al.* [174]

Table 3: Continued

Study type	Cell type or specimen	Test meals	Time frame	No. of subjects	Carotenoids investigated	Aspects studied	Main effects	Reference
Intervention studies with isolated carotenoids/ Supplements	/	vitamin C (120 mg/d) & E (30 mg/d) & β-carotene (6 mg/d) & Se (100 µg/d) & Zn (20 mg/d) suppl.	7.5 y	13017 individuals	β-carotene	Effect on cancer incidence & all-cause mortality	Decrease in total cancer incidence and all-cause mortality in men but not in women	Hercberg *et al.* [177]
	/	β-carotene (15 mg/d) & Se (50 µg/d) & vitamin E (30 mg/d)	5.25 y	30000 individuals	β-carotene	Effect on cancer incidence	Decrease in total mortality and in cancer mortality.	Blot *et al.* [176]
	/	β-carotene (17.8 mg/d)	3.3 y (mean)	232606 individuals	β-carotene	β-carotene and CVD.	β-carotene supplementation increased slightly the risk to develop CVD.	Bjelakovic *et al.* [169]
	/	β-carotene (4 µg/d) or Lutein 1.5 µg/d	10 y	77126 individuals	β-carotene lutein	Effect of carotenoids on lung cancer.	β-carotene: increased risk of small-cell lung cancer. Lutein supplementation: increased risk of non-small cell lung cancer.	Satia *et al.* [173]
	/	Lutein (20 mg/d)	3 months	65 individuals	lutein	Lutein, serum cytokines, lipid profile.	Supplementation with lutein: decreased levels of IL-6 and MCP-1, also of LDL and TG.	Xu *et al.* [196]
	Plasma	Lycopene (6 or 15 mg/d)	8 wks	126 men	lycopene	Lycopene and endothelial health, ox. stress.	Lycopene supplementation (15 mg/d): increased SOD plasma activity, decreased lymphocyte DNA comet tail length.	Kim *et al.* [183]
	Plasma and urine	Lycopene (0,6.5, 15 and 30 mg/d)	8 wks	77 individuals	lycopene	Lycopene and biomarkers of oxidative stress.	Lycopene supplementation: no effect on urinary F2-isoprostanes, MDA, LDL oxidation. 30 mg/d: decreased lymphocyte DNA damage and urinary 8-OHdG.	Devaraj *et al.* [187]

Table 3: Continued

Study type	Cell type or specimen	Test meals	Time frame	No. of subjects	Carotenoids investigated	Aspects studied	Main effects	Reference
Intervention studies with isolated carotenoids/ Supplements	Serum	Lycopene (10 mg/d)	2 months	35 diabetic patients	lycopene	Lycopene and ox. stress	Lycopene supplementation: no effect on antioxidant capacity, oxidized LDL, but increased MDA.	Neyestani *et al.* [188]
	Plasma	Lycopene (12 mg/d)	56 d	37 woman	lycopene	Lycopene and lymphocyte DNA damage.	Lycopene consumption reduced DNA damage in lymphocytes but did not prevent it.	Zhao *et al.* [189]
	Plasma	Lycopene (15 mg/d)	1 wk	8 individuals	lycopene	Lycopene and lymphocyte DNA damage.	Lycopene consumption prevented DNA damage in lymphocytes.	Torbergsen *et al.* [190]
	Prostate cancer	Lycopene (15 mg/d)	3 wks	26 men	lycopene	Lycopene and prostate cancer.	Lycopene consumption : no effect on apoptotic markers.	Kucuk *et al.* [193]
	Plasma	Lycopene	1 wk	18 men and 9 woman	lycopene	Lycopene and biomarkers of endothelial health.	Lycopene: no effect on MDA levels.	Denniss *et al.* [194]

Abbreviations: n.i., not investigated; suppl, supplements; CHD, coronary heart disease; 8-OHdG= 8-oxo-2’ deoxyguanosine; CAD, coronary artery disease; TG, triglycerides.

3.3.4 Summary and outlook

A large number of animal trials have strongly suggested the implication of NF-κB, Nrf2, and additional transcription factors, such as STAT, in anti-inflammatory effects of carotenoids, and thereby influencing many downstream targets such as cytokine formation. Furthermore, the effects were correlated with results from several studies, several studies focusing on the decreased activation of proinflammatory cell types such as CD4(+), CD8(+), and neutrophils, and possibly even involving (via their metabolites) other nuclear receptors such as RAR. This represents a novel area to be further scrutinized. Although often supraphysiological doses were used to achieve this response, there are also indications that lower and rather chronically applied concentrations are likewise effective, although more studies are needed in this domain. An interesting approach in using *in vivo* models could be the use and development of novel, more water-soluble carotenoids, such as crocin and disodium disuccinate astaxanthin. Disodium disuccinate astaxanthin has been investigated for cardioprotection and has been shown to reduce some inflammatory (and oxidative stress) markers (e.g. prostaglandin F2α [PGF2α], 5-hydroxyeicosatetraenoic acid, 8-isoprostane F2α) in a peritoneal mouse inflammation model, although doses were extremely high (500 mg/kg for 7 days) [158].

3.4 Human studies

3.4.1 Markers of inflammation and oxidative stress and general aspects

Human intervention studies (Table 3), especially when conducted as a randomized, double-blind, and placebo-controlled design, are still considered the "gold standard" in nutritional sciences for testing health effects of dietary compounds. Among other reasons, studies that include disease outcome add much more evidence toward proving a potential relationship between carotenoid consumption and disease incidence. However, with respect to studying secondary plant compounds including carotenoids, human studies are faced with a dilemma. When administering carotenoid-rich foods, such as certain fruits and vegetables, many confounding factors prevail that include dietary fiber, minerals, and vitamins, which are also present in the food matrix. However, when giving individual compounds such as in the form of supplements, synergistic effects between carotenoids and micronutrients (e.g. between vitamin C and vitamin E), aspects of overdosing, or altered bioavailability (missing food matrix aiding e.g. in emulsification) may result in different release kinetics, uptake, and biodistribution than carotenoids bound to the food matrix.

An indirect indication of the potential health benefits of carotenoids may be found in epidemiologic studies, although aspects of inflammation in such studies are not typically assessed. In addition, epidemiologic studies cannot demonstrate causality. Furthermore, due to a large number of confounding factors, it is difficult to predict the effect of individual food constituents. Nevertheless, a number of large-scale prospective cohort studies such as the EPIC study [159], the Los

Angeles Artheriosclerosis Study [160], and the study by Kabagambe *et al.* [161] have produced interesting and robust data with respect to "hard end points." Most of these studies have been reviewed in further detail elsewhere [84,162]. They suggest that there are significant health benefits when consuming 3 to 8 servings of fruits and vegetables per day, whereas a lower number of servings often showed to be ineffective [163,164]. Likewise, several prospective studies have suggested that carotenoid consumption in the diet was associated with a reduced risk of cardiovascular mortality [165], or developing type 2 diabetes [23], prostate cancer [166], and lung cancer [167]. Similar results were also found for tissue levels and chronic diseases [168].

In contrast to epidemiologic studies that assess the relationship of whole fruits/vegetables and their positive effects on chronic diseases, many intervention studies with isolated carotenoids have failed to demonstrate health benefits. Instead, they appear to show that the risk for developing CVD [169], lung cancer [170–173], or stroke [174] increased. However, a few trials have revealed beneficial effects after carotenoid supplementation, especially when given to populations marginally deficient in carotenoids [175–177], highlighting that administration of potential prooxidants to nonhealthy subjects bears several risks.

3.4.2 Carotenoids, oxidative stress, NF-κB, Nrf2, and other pathways

Compared with *in vitro* studies, it is more difficult to measure the antioxidant effects of carotenoids *in vivo*, as many factors govern the antioxidant system [84] and only small or transient effects are expected (Table 3). Pool *et al.* [178] showed that consuming 330 mL tomato juice

(40 mg lycopene) or 330 mL carrot juice (containing 22 mg β-carotene and 16 mg α-carotene) or consuming 10 g dried spinach powder in water/milk resulted in a significant decrease in endogenous levels of strand breaks in lymphocyte DNA. The carrot juice intervention was the only intervention in this study that significantly reduced oxidative damage. In another carrot juice intervention [179], 240 mL carrot juice for 3 weeks provided to breast cancer survivors significantly reduced 8-iso PGF2α, although the study was without a control group. Another study by Rehman *et al.* [180] (5 individuals) showed that a single serving of tomatoes (360-728 g/d) reduced oxidative DNA base damage level in white blood cells within 24 hours. It has also been shown that the intake of tomato products for 21 days decreased urinary 8-iso-PGF2α by 50% [181]. The consumption of lycopene-rich tomato products (sauce, paste, or juice) for 4 to 8 weeks decreased oxidative DNA [182] and lymphocyte DNA damage in healthy patients [183], and it increased the resistance of low-density lipoprotein (LDL) to oxidation in diabetic patients [184], similar to another study [185]. Lycopene-rich tomato paste also protected against cutaneous photodamage by reducing mitochondrial DNA damage [186].

Some positive, but more mixed results were seen in studies including carotenoid supplements. Although taking lycopene (6.5, 15, 30 mg/d) in pure form for 8 weeks had no effect on urinary F2-isoprostanes, MDA, and LDL oxidation rate, 30 mg/d reduced lymphocyte DNA damage and urinary 8-OHdG in healthy subjects [187]. A similar result was found in diabetic patients receiving 10 mg/d of lycopene. The supplementation had no effect on total antioxidant capacity or oxidized LDL, but reduced MDA in serum [188]. Supplementing 12 mg/d lycopene for 56 days

reduced H_2O_2-induced DNA damage in lymphocytes, but it did not prevent DNA damage [189], although supplementation with 15 mg/d lycopene for 1 week prevented H_2O_2-induced DNA damage in lymphocytes [190].

A few studies have investigated carotenoid supplementation and their effect on factors involved in the NF-κB pathway. Lycopene-rich foods have received the most attention, possibly due to the relation to tomatoes and the Mediterranean diet. For example, tomato juice administered for 2 weeks to human subjects reduced IL-2 and IL-4 secretion of peripheral blood mononuclear cells (PBMCs) [191], and the consumption of a tomato-based drink for 26 days lowered TNF-α secretion by 34% [192]. Although these results are promising, other studies with lycopene supplementation or tomato products showed rather limited effects, that is, no effect on apoptotic markers [193], MDA [194], C-reactive protein [195], and nitrite/nitrate [194]. In early arthrosis patients, supplementing 20 mg lutein per day for 3 months reduced plasma IL-6 and MCP-1 and improved serum lipids, when compared with subjects receiving a placebo. This indirectly indicated that NF-κB may have been involved [196]. In subjects receiving enteral nutrition, carotenoid enrichment (3 mg/1500 kcal) reduced NF-κB lymphocyte expression, after 3 months of intervention, as compared with a control group [197].

In contrast, 2 or 8 servings/d of fruits and vegetables for 4 weeks did not show any effect on TNF-α and IL-12 production, and the same was observed for C-reactive protein concentrations. However, consuming more than 8 servings/d reduced C-reactive protein concentration [198]. In overweight or obese postmenopausal woman, consuming 2, 5, or 10

servings of vegetables/d for 3 weeks had no effect on urinary 8-isoprostane F2α or serum C-reactive protein [199], despite a clear increase in carotenoid plasma levels. Perhaps the short time of intervention limited observable effects on the inflammation markers.

Only a few human studies have investigated the relationship of carotenoids and Nrf2 expression. A recent study by Talvas *et al.* [200] showed that cells incubated with sera from men who consumed red tomato paste had a significant up-regulation of insulin-like growth factor binding protein 3 (IGFBP3) and Bax/Bcl-2 ratio and down-regulation of cyclin-D1, p53, and Nrf2. These are all genes implicated in the cell cycle, cell stress response, apoptosis, or cell proliferation. Moreover, the study showed that cells incubated with sera from men who consumed purified lycopene had significant up-regulated IGFBP3, c-fos, and μPAR, which are genes implicated in cell proliferation and carcinogenesis. In another recent study, lycopene supplementation (7 mg/d for 3 weeks) was unable to modulate HO-1 in lymphocytes of young men, perhaps because they were already healthy [201]. Middle-aged, moderately overweight subjects who received a lycopene-rich diet (224-350 mg) or lycopene supplements (70 mg/week) for 12 weeks showed significantly improved serum-amyloid A, which is a marker of systemic and high-density lipoprotein– associated inflammation [202].

3.5 Conclusions, gaps of knowledge, and future research

Many mechanistic (*i.e*. *in vitro* studies) investigating the effect of carotenoids on various markers of oxidative stress and inflammation have indicated beneficial health effects, by influencing transcription factors, such as NF-κB or Nrf2, and their downstream targets, such as IL-8 and PGE-2 or HO-1, SOD, respectively. However, other, less well-studied molecular targets, such as RAR, may also play a role.

Thus, although several pathways possibly related to carotenoids, inflammation, and oxidative stress have been uncovered, many aspects remain poorly understood and require more research. This research should include the synergistic aspects between different carotenoids and other compounds such as vitamins, their dosing, and the role of carotenoid breakdown products or metabolites. Taking carotenoid supplements alone has often failed or even indicated negative effects on disease risk in some trials, especially in subjects at risk for oxidative stress, such as smokers. Although this remains controversial, it may be explained by the absence of synergistic effects with compounds normally present in whole foods, such as other antioxidants like vitamin E or C, [203,204]. Carotenoids, when taken as a supplement and not in a food matrix, especially when taken in significant doses, may act as prooxidants, which would be in line with the effects seen *in vitro* [205].

Future research should investigate which carotenoids and their metabolites, such as apocarotenoids or water-soluble derivatives, may constitute suitable modulators to alter pathways related to oxidative stress and inflammation. Research should also focus on the effects that

other matrix components may have on their metabolism and potential anti-inflammatory properties. Such studies will likely advance understanding into the potential dose-relation effects. Further, studying of molecular targets of this promising group of secondary plant compounds are warranted.

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3. Objectives of the Thesis (Chapter 3):

A diet rich in fruits and vegetables and therefore in phytochemicals is recommended by the World Health Organization (WHO). Polyphenols and carotenoids constitute two important classes of phytochemicals with anti-oxidative and anti-inflammatory properties, and epidemiological studies have been showing that they have also health beneficial effects regarding chronic diseases, such as on diabetes or cardio-vascular diseases. However, there is not much data about their impact on the gut mucosa, and both phytochemical groups could have a positive effect on gut related inflammatory conditions, such as inflammatory bowel diseases (IBD).

Our hypothesis was that polyphenols and carotenoids can have an impact on the gut mucosa by reducing or preventing inflammatory processes. We intended to study this with the help of local fruits and vegetables and with a cell model including Caco-2 cells together with HT-29-MTX cells and THP-1 cells, resembling the intestinal lining (mucosa).

Our approach was to first select local plum and cabbage varieties as they are rich in polyphenols and carotenoids and of high nutritional density. We then investigated their carotenoid and polyphenol and micro-and macronutrient profiles, as well as their contribution to anti-oxidative capacity. These results are presented in Chapter 4.

The second aim was to determine how the carotenoid and polyphenol profile will change during *in vitro* gastro-intestinal digestion and kitchen procedures from their original composition to the

bioaccessible form. For this purpose, we selected varieties with high, low or contrasting carotenoid and polyphenol content. We then investigated the uptake of carotenoids and polyphenols into a Caco-2 monoculture and a co-culture of Caco-2 cells with mucus producing HT-29-MTX cells to determine if the mucus acted as a barrier or not. These results are presented in Chapter 5.

The third aim was to investigate the impact of carotenoids and polyphenols on gastrointestinal health. We therefore used a monoculture of Caco-2 cells and a triple-culture model consisting of a coculture of Caco-2/HT-29-MTX cells and THP-1 cells. We stimulated the cell models with an inflammatory stimulus (a mixture of TNF-α, IL-1β and LPS) and then added the digesta, to investigate the potential of carotenoids and polyphenols to reduce inflammatory responses. Toward this aim, we selected different inflammatory markers including cytokines, chemokines and different cellular signalling cascades. We also investigated their impact on the proteomic response, reflecting a more broader, yet non-targeted approach. These results are presented in Chapter 6 and Chapter 7.

4. Published Research Manuscripts

(Chapter 4-7)

Chapter 4 : Carotenoids, Polyphenols and Micronutrient Profiles of *Brassica oleraceae* and Plum Varieties and their Contribution to Measures of Total Antioxidant Capacity

In this Chapter 4 we investigated the carotenoid and polyphenol content of 27 cabbages and 17 plum varieties, as well as their micro- and macronutrient content and their contribution to anti-oxidant capacity. The results obtained during this work have been resulted in an article with the title: Carotenoids, polyphenols and micronutrient profiles of *Brassica oleraceae* and plum varieties and their contribution to measures of total antioxidant capacity. Anouk Kaulmann, Marie-Caroline Jonville, Yves-Jacques Schneider, Lucien Hoffmann, Torsten Bohn. Food Chem. 2014 Jul 15; 155: 240–250.

Carotenoids, Polyphenols and Micronutrient Profiles of *Brassica oleraceae* and Plum Varieties and their Contribution to Measures of Total Antioxidant Capacity

Abstract:

The consumption of phytochemicals such as carotenoids and polyphenols within whole fruits and vegetables has been associated with decreased incidence of various inflammation and oxidative stress related chronic diseases, which may be due to direct antioxidant effects, or indirect mechanisms such as affecting signal transduction/gene expression. Within the present study, we investigated the antioxidant composition of two major groups of vegetables and fruits, *Brassica oleraceae* and *prunus* spp., and estimated their contribution to antioxidant capacity. For this purpose, 17 plum and 27 *Brassica* varieties were collected in Luxembourg, and analysed for their individual polyphenol and carotenoid profile, vitamin C, dietary fibre, and minerals/trace elements, and their correlation with markers of antioxidant capacity (FRAP, ABTS, Folin–Ciocalteu). Total carotenoid and polyphenol content varied considerably between the different *Brassica* and plum varieties, with highest concentrations in the variety Kale (13.3 ± 0.58 mg/100 g wet weight) and Cherry plum (1.96 ± 0.28 mg/100 g) for carotenoids; and Kale (27.0 ± 0.91 mg/100 g) and Kirks plum (185 ± 14 mg/100 g) for polyphenols. In developed multiple linear-regression-models for *Brassica*, flavonoids, anthocyanins, lutein and vitamin C were found to be the best predictors of antioxidant capacity as assessed by FRAP (R^2 = 0.832) and flavonoids, neochlorogenic acid and vitamin C as assessed by ABTS (R^2 = 0.831); while for plums these were selenium, total sugars, chlorogenic acid and vitamin C (R^2 = 0.853), and selenium, chlorogenic acid and flavonoids for FRAP (R^2 = 0.711). When considering *Brassica* and plum consumption in Luxembourg, it

is estimated that both contribute to an antioxidant intake equivalent to 26 and 6 mg per day of ascorbic acid equivalents, respectively.

4.1 Introduction

It is generally recommended by health organizations such as the WHO that fruits and vegetables should be frequently incorporated into the diet as part of a healthy lifestyle. Several epidemiological studies have shown that the long term intake of fruits and vegetables is significantly correlated with a decreased risk of developing inflammation and oxidative stress related chronic diseases, including cardiovascular diseases (D'Odorico *et al.*, 2000), type 2 diabetes (Bazzano, Li, Joshipura, & Hu, 2008) and cancer (Eliassen *et al.*, 2012). Fruits and vegetables, also in form of processed products (*i.e*. tea, coffee, chocolate, and red wine) contain significant amounts of various phytochemicals, which have been postulated to provide health beneficial effects, reducing the risk of chronic diseases. There exist far over >10,000 different phytochemicals such as carotenoids, polyphenols, and phytosterols. However until today, it is poorly comprehended which of these are the most bioactive with respect to the attributed health effects. Carotenoids and polyphenols constitute 2 predominant classes of phytochemicals, both possessing properties that have been related to health benefits, such as via impacting cellular signalling cascades or their antioxidant properties (Palozza, Serini, Ameruso, & Verdecchia, 2012). Carotenoids are, together with terpenes, the most predominant fat-soluble secondary plant metabolites. Over 700 carotenoids have been identified, but only around 100 play a role in the daily diet (Bohn, 2008). Polyphenols represent the largest group of water-soluble phytochemicals. As exogenous antioxidants, both take part in antioxidant defense

mechanisms, preventing damaging effects of reactive oxygen species (ROS) on DNA, proteins, and lipids. While carotenoids are involved in the scavenging of singlet molecular oxygen ($^1O_2$) and peroxyl radicals, especially in the lipid bilayer, the antioxidant capacity of polyphenols depends on their ability to scavenge either free radicals or lipid peroxyl radicals as well as by acting as singlet oxygen quenchers (Bouayed & Bohn, 2010), predominantly in aqueous compartments such as the cytosol. More recently, several studies have demonstrated that polyphenols and carotenoids significantly down-regulated the expression of pro-inflammatory cytokines, possibly due to alterations of the NF-κB pathway (Kim, Seo, & Kim, 2011; Romier, Van de Walle, During, Larondelle, & Schneider, 2008; Vazquez-Agell *et al.*, 2013), and impacted Nrf2, a transcription factor related to the expression of detoxifying agents such as phase II enzymes, meaning that they might act, in addition to directly quenching ROS, as indirect antioxidants, which may in fact be their primary activity *in vivo*. Carotenoid and polyphenol intake and serum concentrations have been inversely associated with reduced incidence of chronic diseases such as type 2 diabetes (Coyne *et al.*, 2005). Due to negative results following intervention studies with isolated compounds (de Maat, Pijl, Kluft, & Princen, 2000; Hininger *et al.*, 2001), it has been argued that the effects rest rather in the entire plethora of phytochemicals and/or micronutrients present in fruits or vegetables, rather than the consumption of an individual phytochemical. Carotenoids are found at high concentrations in a variety of coloured vegetables and some fruits, ranging from 0.1 to >2 mg/100 g. In a normal diet, humans consume approx. 4–15 mg carotenoids per day and capita (Biehler *et*

al., 2012). By contrast, the daily intake of polyphenols ranges 2–3 magnitudes higher, up to 1 g per day per capita (Scalbert & Williamson, 2000). Nevertheless, due to their low absorption and high metabolism, plasma carotenoid concentrations are several µmol/L (Bohn, 2008), while those of polyphenols may be lower (Scalbert & Williamson, 2000). However, the production of local fruits and vegetables plays an integral part for supplying healthy foods rich in micronutrients and phytochemicals. Luxembourg has been putting much emphasis on the production of regional products, but only 1% of fruits and vegetables consumed in Luxembourg are also produced there (Ministère de l'Agriculture, 2009). In 2004, 8.328 tons of fruits (Le portail des Statistiques, 2012b) and 2.750 tons of vegetables (Le portail des Statistiques, 2012a) have been commercialized in Luxembourg, with *Prunus* species (including plums and mirabelles) representing 7% of the produced fruits, second only to apples, with a consumption (as fresh fruits) of 16 kg/year. For vegetables, *Brassica oleraceae* species such as broccoli, cabbage, turnip and cauliflower represent ca. 3% of the produced vegetables, ranking eighth among the consumed vegetables (13.8 kg/year). Both *Brassica oleraceae* and plum can contain relatively high concentrations of carotenoids and polyphenols, e.g. ca. 2 mg/100 g (Biehler *et al.*, 2012) and 30–60 mg/100 g, respectively, in kale (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004), and 0.4 mg/100 g and 115 mg/100 g, respectively, for plums (Manach *et al.*, 2004; Souci, Fachmann, & Kraut, 2000). In the present study, we estimated the contribution of present phytochemicals (carotenoids and polyphenols), selected micro- and macronutrients to antioxidant activity and further estimated the

contribution of *Brassica oleraceae* and plum consumption to the intake of antioxidants.

4.2 Materials and methods

4.2.1 Chemicals and standards

All products were of analytical grade or higher. 18 MΩ water was prepared with a purification system from Millipore (Brussels, Belgium) and was used throughout. Unless otherwise stated, all chemicals including aluminium chloride ($AlCl_3$), 2-2´-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2-2´-azobis (2-methylpropionamidine) dichloride (AAPH), (+)-catechin, gallic acid, Folin–Ciocalteu's phenol reagent, alpha-amylase, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), ascorbic acid (vitamin C) and 2,6 dichlorophenolindophenol were procured from Sigma–Aldrich (St. Louis, MO, USA). Iron (III)chloride 6-hydrate, sodium hydroxide (NaOH), sodium acetate, acetone, cetyl trimethylammonium bromide (CTAB), sodium carbonate (Na_2CO_3) and potassium chloride were obtained from Merck (Darmstadt, Germany). Acetic acid, hydrogen chloride (HCl), ammonium acetate, sulphuric acid (H_2SO_4), sodium nitrite ($NaNO_2$) and oxalic acid were from VWR (Leuven, Belgium). Methanol was procured from Biosolve (Valkenswaard, The Netherlands). Nitric acid (HNO3) was procured from SCP Science (Courtaboeuf, France). Carotenoid standards of b-carotene, b-cryptoxanthin and lutein were purchased from Extrasynthèse (Lyon, France), a-carotene, violaxanthin, neoxanthin, phytoene and phytofluene were purchased from CaroteNature (Lupsingen, Switzerland), trans-b-apocarotenal was from Sigma–Aldrich. Certified

purity of all standard was above 95%. Polyphenol standards, *i.e*. (+)-catechin, caffeic acid, p-coumaric acid, cryptochlorogenic acid (4-caffeoylquinic acid), neochlorogenic acid (3-caffeoylquinic acid), quercetin, quercetin 3-O-galactoside, kaempferol, kaempferol 3-O-glucoside, ferulic acid, syringic acid, sinapic acid, gallic acid and vanillic acid were obtained from Sigma–Aldrich. Chlorogenic acid and cinnamic acid were purchased from Merck. Kaempferol-3-O-rutinoside was obtained from Extrasynthèse and 4-hydroxybenzoic acid was purchased from Thermo Fisher Scientific (Geel, Belgium).

4.2.2 Sample preparation

Twenty-seven *Brassica oleraceae* varieties and 17 plum varieties were procured from different local Luxembourgish farmers or markets (Table 1) between September 2010 and November 2011. *Brassica* varieties (n = 1 piece per variety except for Brussels sprouts (n = 4–6), and cauliflower and broccoli (n = 2–3) were purchased fresh from markets, and stored for analysis for a maximum of 2 weeks prior to further processing. Plums were likewise obtained in fresh form from various markets, and aliquots of 6–9 plums per variety were chosen for further processing, with storage at a maximum of 1 week at 4 °C prior to processing. For each *Brassica* variety, all edible leaves were carefully separated, packed into transparent polyethylene bags, dipped in liquid nitrogen and stored at -80 °C. For each plum variety, the plums were cut into half with a sharp knife, the kernel removed, packed into transparent polyethylene bags, dipped in liquid nitrogen and stored at -80 °C. Samples were then further lyophilised (Christ freeze dryer, Thermo Fisher Scientific, Geel, Belgium) during 24 h, combined and homogenized with a grinder (Mortar Grinder RM 200,

Retsch, Aartselaar, Belgium) and stored in 50-mL plastic centrifuge tubes at -20 °C until analysis.

4.2.3 Extraction of phenolic compounds

For the extraction of phenolics a protocol similar as described earlier was followed (Bouayed, Hoffmann, & Bohn, 2011). Three independent extractions for each variety were carried out on ice under dim light. For this objective, 1 g of lyophilized fruit or vegetable powder was weighted into a 15 mL screw-cap tube and mixed with 7.5 mL of 80% (v/v) methanol. The mixture was then sonicated for 20 min and centrifuged for 5 min at 4000g (Heraeus Multifuge X3, Thermo Scientific, Leuven). The supernatant was recovered and the residue was re-extracted with 3 mL of 100% methanol, sonicated for 10 min and centrifuged for 5 min at 4000g. This step was repeated once more. The solvent of the combined extract was then removed under a stream of nitrogen in a Turbovap blower (Caliper Life Sciences, Teralfene, Belgium) until ca. 2 mL remained, then 2 mL of 100% methanol were added and the final volume noted down. The extracts were stored under argon at -80 °C until further analysis.

4.2.4 Extraction of carotenoids

For the extraction of carotenoids the original protocol of Biehler, Kaulmann, Hoffmann, Krause, and Bohn (2011) was adapted. All procedures were carried out on ice and in the dark as far as possible. In short, 250–500 mg of weighed sample were mixed with 6 mL of methanol, put on ice for 15 min, and then centrifuged for 5 min at 2500g (4 °C) (n = 3). The supernatant was transferred into a 50 mL centrifuge tube. The residue was re-extracted first with 8 mL of

hexane:acetone (1:1, v/v) and then 4 mL of hexane, each centrifuged for 2 min at 2500g, and supernatants were combined with the first methanol phase. Then, 10 mL of a saturated sodium chloride were added. The mixture was mixed and centrifuged for 2 min at 2500g. The upper phase was transferred into a new 50 mL centrifuge tube, and the lower phase was re-extracted with additional 5 mL of hexane and combined with the first hexane phase. Then, a 10 mL aliquot of the combined plum extract and 5 mL of the combined *Brassica* extract were transferred into a 15 mL centrifuge tube and the solvent was evaporated under a stream of nitrogen. The residue was redissolved in 600 µL of MTBE: methanol (3:7, v/v), transferred to an amber HPLC vial and stored at -80 °C until analysis for no more than 1 week.

4.2.5 UPLC-DAD analysis of carotenoids

For the carotenoid quantification an Acuity UPLC BEH C18 column (Waters Inc., Zellik, Belgium, 2.1 x 100 mm, 1,7 µm particle size, set at 40 °C) was used in combination with a Waters Acquity UPLC system (Milford, MA) equipped with a photodiode array detector. The eluents were (A) ammonium acetate (50 mMol) in water (60%:40%, v/v) and (B) acetonitrile: dichloromethane (80%:20%, v/v) and the gradient was as follows: 0 min, 42% B, flow rate 0.35 ml/min; 4 min, 47% B; 13 min, 80% B, flow rate 0.40 ml/ min; 18 min, 85% B; 29 min, 85% B, flow rate 0.35 ml/min; 30 min 42% B. The injection volume was 5 µL. Carotenoids were detected at 286 nm (phytoene), 440 nm (neoxanthin, violaxanthin), 450 nm (lutein, β-carotene and α-carotene) or 455 nm (zeaxanthin and b-cryptoxanthin) according to their absorption maximum. For quantification, 7 point – linear

calibration curves were prepared with external standards for each compound, with concentrations ranging from 0.01 to 25 µg/mL.

4.2.6 UPLC-DAD analysis of polyphenols

The method was adapted from Deusser, Guignard, Hoffmann, and Evers (2012). For the quantification of polyphenols, a Waters Acquity UPLC® system equipped with a photodiode array detector was used. For separation, an Acquity UPLC® HSS T3 column (1.8- µm particle size, 2.1 x 100 mm) with a flow rate of 0,75 mL/min at 50 °C was used. The eluents were 0.1% (v/v) formic acid in water (A) and 0.1% formic acid in acetonitrile (B) and the gradient was as follows: 0 min, 5% B; 9.27 min, 5% B; 13.53 min, 14% B; 22.60 min, 35% B; 23 min, 95% B; 25 min, 95% B; 26 min, 5% B. The injection volume was 10 µL. Polyphenols were detected at 254, 280, 300 or 320 nm according to their absorption maximum. For quantification, 7 point – linear calibration curves were prepared with external standards for each compound, with concentrations ranging from 0.01 to 50 mg/L. Samples with higher concentrations were appropriately diluted with water/methanol (90:10 v/v). The sum of polyphenols (UPLC) was detected as the sum of the 18 individual polyphenols (see Section 2.1).

Table 1: *Brassica oleraceae* and plum varieties investigated in the present study

Food item	Latin Term	Locality	Time of obtainment
Pointed cabbage	*Brassica oleracea var. capita f. acuta*	Kockelscheuer (LUX)	Autumn 2010
Red cabbage	*Brassica oleracea convar. capitata var. rubra*	Kockelscheuer (LUX)	Autumn 2010
White cabbage	*Brassica oleracea convar. capitata var. alba*	Kockelscheuer (LUX)	Autumn 2010
Subaro (red cabbage var.)	*Brassica oleracea convar. capitata var. rubra*	Everlange (LUX)	Autumn 2010
Kalorama (white cabbage var.)	*Brassica oleracea convar. capitata var. alba*	Everlange (LUX)	Autumn 2010
Reguma (red cabbage var.)	*Brassica oleracea convar. capitata var. rubra*	Everlange (LUX)	Autumn 2010
Marner Lagerrot (red cabbage var.)	*Brassica oleracea convar. capitata var. rubra*	Everlange (LUX)	Autumn 2010
Marner Lagerweiss(white cabbage var.)	*Brassica oleracea convar. capitata var. alba*	Everlange (LUX)	Autumn 2010
Savoy cabbage	*Brassica oleracea convar. capitata var. sabauda*	n.i	Autumn 2010
Scots Kale	*Brassica oleracea var. sabellica*	Minsbech (LUX)	Autumn 2010
German Turnip	*Brassica oleracea var. gongylodes*	Minsbech (LUX)	Autumn 2010
Kale	*Brassica oleracea var. sabellica*	n.i	Autumn 2010
Brussels sprouts	*Brassica oleracea var. gemmifera*	n.i	Autumn 2010
Duchy (pointed cabbage var.)	*Brassica oleracea var. capita f. acuta*	Dalheim (LUX)	Summer 2011
Toughma (white cabbage var.)	*Brassica oleracea convar. capitata var. alba*	Bastendorf(LUX)	Summer 2011
Broccoli var. Verde Calabrese	*Brassica oleracea var. italica*	Dalheim (LUX)	Summer 2011
Broccoli var. Marathon	*Brassica oleracea var. italica*	Allerborn (LUX)	Summer 2011
Cauliflower var. White Rock	*Brassica oleracea var. botrytis*	Dalheim (LUX)	Summer 2011
Gigant DDR/Superschmelz (german turnip var.)	*Brassica oleracea var. gongylodes*	Senningerberg (LUX)	Summer 2011
Lanro (german turnip var.)	*Brassica oleracea var. gongylodes*	n.i	Autumn 2011
Marner Lagerrot (red cabbage var.)	*Brassica oleracea convar. capitata var. rubra*	Kockelscheuer (LUX)	Autumn 2011
Marner Lagerweiss (white cabbage var.)	*Brassica oleracea convar. capitata var. alba*	Kockelscheuer (LUX)	Autumn 2011
Dottenfelder Dauer (white cabbage var.)	*Brassica oleracea convar. capitata var. alba*	Steinsel (LUX)	Autumn 2011
Winterfürst (savoy cabbage var.)	*Brassica oleracea convar. capitata var. sabauda*	Kockelscheuer (LUX)	Autumn 2011
Granat (red cabbage var.)	*Brassica oleracea convar. capitata var. rubra*	Steinsel (LUX)	Autumn 2011
Endivie	*Cichorium endivia*	n.i	Autumn 2010
Radicchio	*Cichorium intybus var. foliosum*	n.i	Autumn 2010

Table 1: Continued

Food item	Latin Term	Locality	Time of obtainment
Centenar 1(plum var.)	*Prunus domestica*	Ernster (LUX)	Summer 2011
Ersinger (plum var.)	*Prunus domestica*	Ernster (LUX)	Summer 2011
Orthenauer (plum var.)	*Prunus domestica*	Ernster (LUX)	Summer 2011
Centenar 2(plum var.)	*Prunus domestica*	Ernster (LUX)	Summer 2011
Duederer (plum var.)	*Prunus domestica*	Wellenstein (LUX)	Summer 2011
Green Gage plum	*Prunus domestica subsp. italica var. claudiana.*	Schwebsange (LUX)	Summer 2011
Plum 620 [a]	*Prunus domestica*	Beiler (LUX)	Summer 2011
Plum 605 [a]	*Prunus domestica*	Lullange (LUX)	Summer 2011
Plum 575 [a]	*Prunus domestica*	Marnach (LUX)	Summer 2011
Kuhpanz (plum var.)	*Prunus domestica*	Hupperdange (LUX)	Summer 2011
Mirabelle plum	*Prunus domestica subsp. syriaca*	Karelshaff (LUX)	Summer 2011
Graf Althanns	*Prunus domestica subsp. italica var. claudiana.*	Ernster (LUX)	Summer 2011
Kirks plum	*Prunus domestica*	Bech (LUX)	Summer 2011
Italian plum	*Prunus cocomilia*	Bech (LUX)	Summer 2011
Normal plum	*Prunus domestica*	Ernster (LUX)	Autumn 2011
President (plum var.)	*Prunus domestica*	Ernster (LUX)	Autumn 2011
Cherry plum	*Prunus cerasifera*	Bettembourg (LUX)	Autumn 2011

n.i. = no information on locality
[a] plums which have not yet been identified

4.2.7 Spectrophotometric analyses of total phenolics, flavonoids, and total anthocyanins

4.2.7.1 Determination of total phenolics

Total phenolic content was determined with Folin–Ciocalteu's phenol reagent as described earlier (Bouayed *et al.*, 2011), using a spectrophotometric analysis (DU800 UV/Visible spectrophotometer, Beckman Coulter, Palo Alto, CA). In short, 1 mL of sample (appropriately diluted phenolic extracts with 50% methanol) or standard was added to a 50 mL centrifuge tube containing 9 mL of water. One millilitre Folin–Ciocalteu's phenol reagent was added and the mixture was shaken. After 5 min, 10 mL of 7% (w/v) Na_2CO_3 was added and the volume was immediately diluted to 25 mL with water and mixed thoroughly. After an incubation of 90 min at room temperature, the absorbance was determined at 750 nm vs. the prepared blank (1 mL 50% methanol instead of sample). The total phenolic content expressed as gallic acid equivalents (GAE) per 100 g fresh weight, was calculated using an external calibration curve (gallic acid, n = 6 concentrations between 0 and 100 mg/L).

4.2.7.2 Determination of total flavonoids

The quantification of total flavonoid content was evaluated with a colorimetric assay (Bouayed *et al.*, 2011). In short, 1 mL of sample (appropriately diluted phenolic extracts) or standard was added to a 15 mL centrifuge tube containing 4 mL of water. At the onset of the experiment, 0.3 mL of 5% (w/v) $NaNO_2$ was added and the mixture was shaken. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At 6 min, 2 mL of 1 M NaOH was added and the volume was immediately diluted

to 10 mL with water and mixed thoroughly. The absorbance vs. the prepared blank was read at 510 nm. The total flavonoid content expressed as catechin equivalents (CE) per 100 g fresh weight was calculated using an external calibration curve (catechin, n = 6 concentrations between 0 and 100 mg/L).

4.2.7.3 Determination of total anthocyanins

The measurement of total anthocyanin content was carried out by the pH differential method (Bouayed *et al.*, 2011). Briefly, 1–5 mL of the phenolic extract was diluted between 2 and 100 times with 2.5 µM potassium chloride solution (pH 1) and 0.4 M sodium acetate buffer (pH 4.5), respectively. After 15 min of incubation, the absorbance was measured at 520 and 700 nm vs. the blank (water). The content of total anthocyanins was expressed as mg cyanidin 3-glucoside equivalents (CGE) per 100 g fresh weight. A molar absorption coefficient of 26,900 L mol^{-1} cm^{-1} (cyanidin 3-glucoside) was used to calculate the concentration of anthocyanins in solution.

4.2.8 Methods to estimate antioxidant capacity

4.2.8.1 ABTS-radical scavenging capacity assay

The antioxidant capacity was measured by the vitamin C equivalent antioxidant capacity (VCEAC) test (Bouayed *et al.*, 2011). In brief, 2.5 mM ABTS and 1 mM AAPH were mixed in PBS (pH 7.4). The mixture was heated in a water bath at 68 °C for ±15 min until the colour of the mixture turned blue-green. The absorbance of this blue-green ABTS radical solution was adjusted to 0.650 ± 0.020 with additional PBS. Then, 40 µL of appropriately diluted sample or

standard were mixed with 1960 µL of ABTS radical solution and incubated in the dark at 37 °C for 10 min. After 10 min, the absorption was determined at 734 nm vs. the blank. The antioxidant capacity expressed as vitamin C equivalents (VCE) per 100 g fresh weight was calculated using an external calibration curve (vitamin C, n = 5 concentrations between 0 and 0.2 mg/L).

4.2.8.2 Ferric-reducing antioxidant power assay (FRAP)

The FRAP method as described earlier was employed (Bouayed *et al.*, 2011). Briefly, the FRAP reagent was prepared freshly by mixing acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron(III)chloride solution (10:1:1, v/v/v) and warmed up to 37 °C before use. Then, 50 µL of sample (appropriately diluted extracts or standard) was mixed with 1.5 mL of FRAP reagent. After an incubation of 4 min the absorbance was determined at 593 nm vs. a blank. The antioxidant capacity expressed as µmol Fe(II) per 100 g fresh weight was calculated using an external calibration curve (iron(II)sulphate solution, n = 7 concentrations between100 and 2000 µmol).

4.2.9 Determination of minerals, trace elements and total soluble solids (TSS)

Samples, 500 mg (dry weight), were digested in 7 mL HNO_3 (plasma pure 67–70%; SCP Science, Courtaboeuf, France) and 3mL H_2O_2 (30% w/w for metal traces analysis; Fisher Scientific, Tournai, Belgium). Acid digestion was performed in Teflon tubes in a microwave oven (Anton Paar Multiwave 3000, Graz, Austria) by increasing temperature and pressure until 200 °C and 30 bar. At the end of the procedure, samples were diluted with H_2O up to 25 mL and

kept at 4 °C prior to analysis. Blank and certified reference material (spinach from China National Analysis Center, NCS ZC73013) were included at each mineralization cycle for quality control. Samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer Elan DRC-e, Waltham, MA, USA) according to the manufacturer's recommendations, analogous as described by Neubauer and Wolf (2004). TSS is an index of soluble sugar content in fruits. TSS (°Brix) in plum varieties was determined with a refractometer (ABBE Mark III, Reichert, Seefeld, Germany), similar as described earlier (Javanmardi & Kubota, 2006).

4.2.10 Ascorbic acid measurements

To measure the ascorbic acid content, Tillmanns' method was used. For titer determination, 20 mL of 0.2% (w/v) oxalic acid were mixed with 0.2 mL of ascorbic acid standard solution (2 mg/mL) and the solution was then titrated with 2,6 – dichlorophenol-indophenol (DI). For sample analysis, 200–250 mg (dry weight) of samples were weighed into a 15 mL centrifuge tube and 4–5 mL of 2% oxalic acid were added. Samples were vortexed for 3 min and centrifuged for another 2 min, the supernatant was then transferred into a new 15 mL centrifuge tube. For the determination of vitamin C content, 2 mL of sample was mixed with 20 mL of 2% oxalic acid and titrated with DI solution. The total amount of vitamin C in mg/ 100 g was calculated from the amount of DI used and by the titre.

4.2.11 Statistics

Unless otherwise stated, all data represent mean ± standard deviation (SD). All data was evaluated by SPSS vs. 19.0 (IBM, Chicago, IL). Equality of variance and normal distribution of results were verified

by box plots and Q–Q plots, respectively. Data was log transformed to achieve a normal distribution. Correlations between antioxidant capacity (FRAP, ABTS) and individual constituents were done by Pearson correlation coefficients. A P-value <0.05 (2-sided) was considered as statistically significant. For predicting antioxidant capacity, a multiple linear regression model was constructed with either FRAP or ABTS as the observed variable, and individual plum and *Brassica* constituents normally linked with antioxidant capacity such as ascorbic acid, total carotenoids, total polyphenols, total flavonoids, total anthocyanin and major individual polyphenols and carotenoids as independent variables. Both stepwise elimination of variables (if P >> 0.10) as well as stepwise insertion of variables (if P < 0.10) was done in order to determine the best fitted models.

4.3 Results

4.3.1 Total carotenoid content

Of the 27 *Brassica oleraceae* varieties, Kale (13.3 mg/100 g), Duchy (8.11 mg/100 g), Broccoli verde (4.69 mg/100 g), Broccoli marathon (3.46 mg/100 g) and Brussels sprouts (3.95 mg/100 g) were the richest in total carotenoids; whereas Reguma (55 µg/ 100 g), Cauliflower (75 µg/100 g) and Dottenfelder (103 µg/100 g) contained only low concentrations of carotenoids (Table 2). For the plum varieties, Cherry plum (1.96 mg/100 g), Kirks plum (1.95 mg/100 g), Italian plum (1.90 mg/100 g) and Graf Althans plum (1.24 mg/100 g) were richest in total carotenoids, while Centenar 1 (247 µg/100 g), Centenar 2 (272 µg/100 g) and Duederer (295 µg/100 g) ranked lowest in total carotenoids. Thus, *Brassica oleraceae* varieties mainly contained

beta-carotene and lutein, violaxanthin, neoxanthin and beta-cryptoxanthin, while plum varieties mainly contained beta-carotene, lutein, alpha-carotene, betacryptoxanthin, zeaxanthin and phytoene.

4.3.2 Polyphenolic compounds

Regarding the total content of *Brassica* polyphenols quantified by UPLC-DAD, Kale showed the highest concentration with 27 mg/100 g and Lanro showed the lowest one with only 95 µg/ 100 g. The highest concentration of total plum polyphenols was found in Kirks plum with 151 mg/100 g while the Cherry plum showed the lowest one with 14 mg/100 g. Neochlorogenic acid, an isomer of chlorogenic acid was the most predominant hydroxycinnamic acid found in the *Brassica* varieties followed by sinapic acid, found in the majority of varieties (Table 3). Chlorogenic acid was found in 3 green *Brassica* varieties but showed highest concentrations in the Chicorium species. A second isomer of chlorogenic acid, *i.e*. cryptochlorogenic acid, was found in 5 red and 3 green *Brassica* varieties. Neochlorogenic acid correlated well with the antioxidant capacity measured via the ABTS test ($R = 0.721$, $P < 0.01$). Chlorogenic acid and its isomer neochlorogenic acid were the most predominant polyphenols found in almost all our plum varieties. Cryptochlorogenic acid, another isomer of chlorogenic acid, was found in six different plum varieties. Among the other hydroxycinnamic compounds, p-coumaric acid as well as ferulic acid was only detected in the Kirks plum, and sinapic acid was not detected in our plum varieties. None of the individual polyphenols correlated significantly with any of the measurements related to antioxidant capacity (FRAP, ABTS).

4.3.3 Total phenolic content

The total phenolic content (TP) in *Brassica* and plums as determined by the Folin–Ciocalteu test ranged from 5 to 209 mg gallic acid equivalents (GAE)/100 g fresh weight (FW) (Table 4). There were large differences in the TP of the different *Brassica* varieties, with the lowest concentrations for white *Brassica* varieties (5.4– 61.5 mg GAE/100 g FW) and highest concentrations for red and green *Brassica* varieties (13–139 mg GAE/100 g FW). In plums there were also large differences observed, with the lowest concentrations for normal plums (61.9 mg GAE/100 g FW) and the highest concentrations for Kuhpanz (209 mg GAE/100 g FW). Plum varieties generally showed higher TP than *Brassica* varieties. When comparing total phenolic content by Folin–Ciocalteu with the sum of individual polyphenols determined by HPLC in *Brassica*, a good correlation ($R = 0.669$, $P < 0.01$) was found.

4.3.4 Total flavonoids (TF) and anthocyanins (TA)

On the basis of fresh weight, TF were highest in red and green *Brassica* varieties (5.5–65.0 mg catechin equivalents, CE/100 g FW), and lowest in white *Brassica* varieties (0.8–27.1 mg CE/100 g FW, Table 4). There were differences in the TF of the different plum varieties with the lowest concentration in normal plums (25.0 mg CE/100 g FW) and the highest concentration in Green gage plums (144 mg CE/100 g FW). Plum varieties generally showed higher TF than *Brassica* varieties. The total anthocyanin content (TA) was highest in red *Brassica* varieties (21.8–71.0 mg cyanidin-3-glucoside

equivalents (CGE)/ 100 g FW) (Table 4). White and green *Brassica* varieties showed lowest TA with 0.3–6.5 mg (CGE)/100 g FW. As expected, correlations between flavonoids and total phenolics ($R = 0.877$, $P < 0.01$) and anthocyanins and total phenolics ($R = 0.679$, $P < 0.01$) were high, as well as their correlation to FRAP ($R = 0.763$ for TF and $R = 0.686$ for TA). The correlation of TF to ABTS was high ($R = 0.882$, $P < 0.01$) and for TA and ABTS ($R = 0.457$, $P < 0.05$).

4.3.5 ABTS, FRAP, and contribution of plum and Brassica to antioxidant intake

ABTS followed, in general, concentrations of phenolics and was highest in red and green *Brassica* varieties (Table 4). In white *Brassica* varieties the antioxidant capacity was somewhat lower, fluctuating between 29 and 191 mg (VCE)/100 g FW. The antioxidant capacity of plum varieties was similar to those observed in red and green *Brassica* varieties, ranging from 195 to 386 mg (VCE)/ 100 g FW. The overall trend of total FRAP followed the concentrations of phenolics. Radical-scavenging activity was again highest in red and green *Brassica* varieties (255–1759 µmol Fe(II)/100 g FW) and lowest in white *Brassica* varieties (163–555 µmol Fe(II)/100 g FW) (Table 4). The FRAP values in plums were slightly higher than those of red and green *Brassica* varieties, varying between 587 and 2919 µmol Fe(II)/100 g FW. The total phenolic content as determined by Folin–Ciocalteu was well correlated with ABTS and FRAP both in *Brassica* ($R = 0.867$ and $R = 0.819$, $P < 0.01$) and plum ($R = 0.660$ and $R = 0.826$, $P < 0.01$). FRAP and ABTS values correlated significantly for both *Brassica* and plum varieties ($R = 0.824$ and $R = 0.560$, $P < 0.05$).

Based on the obtained mean ABTS values for plum and *Brassica*, expressed as mg vitamin C equivalents, the contribution of these two plant foods to antioxidant intake was estimated by multiplying these values with the estimated intake of plum and *Brassica* varieties in Luxembourg, as reported in a previous study (Biehler *et al.*, 2012). When considering *Brassica* and plum phytochemical concentrations and linking them to dietary intake, it is estimated that they contribute to a daily carotenoid intake of ca. 0.3 mg and 20 µg, and to a polyphenol intake of 0.7 and 1.2 mg, respectively.

4.3.6 Minerals, ascorbic acid, sugars

Considering both plum and *Brassica* varieties, the vitamin C concentration varied from 4 to 176 mg/100 g FW (Table 4). The ascorbic acid concentration of plum varieties was, on average lower than that of *Brassica*. Vitamin C was well correlated with FRAP ($R = 0.649$, $P < 0.01$) and ABTS ($R = 0.618$, $P < 0.01$) in *Brassica*, but not in plum varieties ($R = 0.01$ for FRAP and $R = 0.26$ for ABTS). The total mineral content of all tested samples ranged from 178 to 1033 mg/100 g FW (Supplemental data, Table 1), with highest concentrations for Brussels sprouts and Kale. For the plum varieties the highest concentrations were found for the Green gage plum and the Cherry plum. Similar patterns were observed for the micro-minerals, with highest trace elements concentration of *Brassica* varieties found in Brussels sprouts (9.16 mg/100 g) and lowest in Endive (0.18 mg/100 g), and highest and lowest iron concentrations in plums in Kuhpanz (290 µg/100 g) and Kirks plum (80.0 µg/100 g), respectively (Supplemental data), while selenium patterns were

somewhat different. Selenium concentrations were well correlated with ABTS and FRAP in plums ($R = 0.817$, $P < 0.01$ and $R = 0.562$, $P < 0.05$, respectively). The total sugar content in plums (Table 5) varied between 8.5 and 19.6/100 g FW and correlated significantly with ABTS ($R = 0.782$, $P < 0.01$). TSS in cabbage was not investigated due to their comparably lower TSS concentrations (between 0.540/100 g and 4/100 g FW (Rosa, David, & Gomes, 2001; Souci *et al.*, 2000)) compared to plum TSS concentrations.

4.3.7 Multivariate linear regression model

The best fitting model for *Brassica* varieties with respect to ABTS prediction ($R^2 = 0.831$, $P < 0.01$) contained ascorbic acid (standardized coefficient = 0.224, P = 0.050), total flavonoid (standardized coefficient = 0.582, P = 0.001) and neochlorogenic acid (standardized coefficient = 0.238, P = 0.100). Similar results were obtained for predicting FRAP ($R^2 = 0.832$, $P < 0.01$) containing ascorbic acid (standardized coefficient = 0.310, P = 0.012), total flavonoids (standardized coefficient = 0.258, P = 0.094), total anthocyanins (standardized coefficient = 0.580, $P < 0.01$) but also lutein (standardized coefficient = 0.272, P = 0.050). For plum varieties, the most promising model for predicting ABTS ($R^2 = 0.853$, $P < 0.01$) contained selenium (standardized coefficient = 0.535, P = 0.005), total sugars (standardized coefficient = 0.405, P = 0.022), chlorogenic acid (standardized coefficient = 0.254, P = 0.055) and ascorbic acid (standardized coefficient = 0.276, P = 0.039). For FRAP however, the most promising model ($R^2 = 0.711$, $P < 0.01$) contained also selenium (standardized coefficient = 0.424, P = 0.039) and

chlorogenic acid (standardized coefficient = 0.384, P = 0.053) but also total flavonoid content (standardized coefficient = 0.386, P = 0.078).

Table 2: Carotenoid profile and content of the different *Brassica oleraceae* and plum varieties investigated[*].

Variety[+]	α-carotene		β-carotene		β-cryptoxanthin		lutein		violaxanthin		neoxanthin		phytoene		zeaxanthin		Total Carotenoids	
	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD
Pointed cabbage[1]	-	-	338	78.8	746	203	454	127	90.6	24.2	31.9	11.2	-	-	-	-	1660	254
White cabbage[1]	-	-	109	1.55	215	19.6	132	11.9	43.9	3.77	6.38	1.00	-	-	-	-	505	23.3
Kalorama[1]	-	-	22.1	1.62	54.7	3.52	38.5	2.37	16.0	0.92	2.14	0.46	-	-	-	-	134	4.66
Marner Lagerwhite 1[1]	-	-	27.9	1.21	69.9	4.38	47.0	1.60	17.6	0.38	3.35	0.98	-	-	-	-	166	4.93
Endive[1]	-	-	539	26.0	2.49	0.47	1162	70.1	302	8.08	66.5	3.30	-	-	-	-	2072	75.3
German turnip[1]	-	-	82.2	5.75	185	15.1	117	12.1	46.0	3.22	6.48	0.24	-	-	-	-	437	20.5
Brussels sprouts[2]	-	-	718	51.2	1163	81.2	1163	72.4	784	42.2	126	9.73	-	-	-	-	3954	128
Duchy[1]	-	-	2055	96.3	13.8	3.95	4225	180	1474	54.0	346	10.8	-	-	-	-	8114	211
Toughma[1]	-	-	25.5	0.84	76.6	7.10	70.7	4.98	39.2	2.15	10.3	0.61	-	-	-	-	222	9.00
Cauliflower[3]	-	-	22.4	7.82	15.7	2.44	28.9	2.27	6.49	0.77	1.00	0.88	-	-	-	-	74.6	8.58
Gigant DDR/Superschmelz[1]	-	-	49.1	10.5	97.3	20.8	66.6	14.0	36.0	5.47	8.08	1.81	-	-	-	-	257	27.8
Lanro[1]	-	-	40.7	3.06	97.9	7.49	54.3	4.92	40.7	3.15	7.65	0.70	-	-	-	-	241	10.0
Marner Lagerwhite 2[1]	-	-	20.3	1.60	50.5	4.71	32.5	2.40	14.8	0.63	6.16	0.81	-	-	-	-	124	5.62
Dottenfelder[1]	-	-	11.8	1.33	40.7	4.10	29.8	2.75	16.0	1.69	5.10	0.67	-	-	-	-	103	5.43
Winterfürst[1]	-	-	67.7	2.75	130	9.13	96.2	5.89	54.2	3.11	14.2	0.96	-	-	-	-	363	11.7
Red cabbage[1]	-	-	38.4	3.50	46.4	3.16	48.3	0.33	26.3	1.55	3.24	0.86	-	-	-	-	163	5.05
Subaro[1]	-	-	22.9	2.19	26.2	5.59	34.5	3.32	30.1	2.11	3.89	0.54	-	-	-	-	118	7.20
Reguma[1]	-	-	9.88	2.00	5.57	0.22	16.5	0.55	20.6	1.56	2.78	0.76	-	-	-	-	55.4	2.71
Marner Lagerred 1[1]	-	-	57.0	16.9	130	33.8	79.5	20.7	38.8	4.41	5.89	1.23	-	-	-	-	311	43.4
Radicchio[1]	-	-	20.7	1.71	3.48	0.29	455	17.5	116	4.36	13.4	0.75	-	-	-	-	608	18.2
Marner Lagerred 2[1]	-	-	49.1	12.1	76.0	20.2	59.8	16.5	35.5	8.05	8.58	2.89	-	-	-	-	229	30.0
Granat[1]	-	-	122	11.3	307	38.1	200	20.3	63.1	4.19	25.6	2.27	-	-	-	-	717	44.9
Scots Kale[1]	-	-	436	68.2	9.78	1.02	433	98.8	117	24.4	7.49	2.59	-	-	-	-	1003	123
Savoy cabbage[1]	-	-	63.2	15.2	5.66	1.61	220	30.4	153	18.6	15.5	1.31	-	-	-	-	457	38.8
Kale[1]	-	-	4400	298	21.5	5.59	6522	481	1706	103	640	77.6	-	-	-	-	13290	580
Brokkoli Verde[3]	-	-	1138	69.5	15.1	1.44	2805	191	564	41.6	164	22.2	-	-	-	-	4686	209
Brokkoli Marathon[3]	-	-	873	224	8.24	6.89	1905	512	538	144	139	38.1	-	-	-	-	3464	579

Table 2:Continued

Variety[+]	α-carotene		β-carotene		β-cryptoxanthin		lutein		violaxanthin		neoxanthin		phytoene		zeaxanthin		Total Carotenoids	
	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD	Mean µg/100g	SD
Centenar 1[4]	-	-	76.2	25.3	4.33	1.40	132	46.6	-	-	-	-	32.7	13.1	1.86	0.57	247	54.7
Ersinger[4]	8.09	2.48	227	16.0	8.03	0.20	193	15.1	-	-	-	-	30.2	1.85	3.63	0.00	470	22.3
Orthenauer[4]	8.66	0.45	171	16.3	5.47	0.59	130	7.99	-	-	-	-	24.7	1.15	2.59	0.00	343	18.2
Centenar 2[4]	8.23	1.12	84.1	9.21	4.66	0.39	147	25.3	-	-	-	-	26.0	3.05	2.05	0.20	272	27.1
Duederer[4]	3.82	0.78	130	5.60	3.17	0.19	141	7.06	-	-	-	-	12.7	3.69	4.68	0.00	295	9.77
Green Gage plum[4]	41.9	6.36	465	56.4	26.2	1.37	269	9.86	-	-	-	-	94.1	10.0	12.9	0.00	909	58.5
Plum 620[4]	7.31	1.01	161	9.27	34.4	3.37	127	16.3	-	-	-	-	227	19.2	6.00	0.31	563	27.1
Plum 605[4]	13.5	0.48	320	18.0	12.2	0.79	145	4.07	-	-	-	-	60.5	1.88	4.52	0.00	556	18.6
Kuhpanz[4]	27.3	1.82	448	40.8	28.4	2.76	217	21.4	-	-	-	-	33.8	8.05	11.8	0.00	766	46.9
Plum 575[4]	13.0	5.07	660	13.7	48.0	2.18	187	8.83	-	-	-	-	74.7	5.40	12.5	0.16	995	18.0
Mirabelle plum[4]	106	57.8	472	211	32.6	17.0	178	47.5	-	-	-	-	95.7	46.5	-	-	884	229
Graf Althanns[4]	54.9	12.0	938	18.3	30.6	0.76	101	8.86	-	-	-	-	111	1.87	5.46	0.00	1242	23.7
Kirks plum[4]	51.6	17.7	1341	184	47.4	4.39	459	58.6	-	-	-	-	45.8	10.8	-	-	1945	195
Italian plum[4]	45.4	14.1	1162	386	73.0	27.1	151	18.1	-	-	-	-	466	155	6.36	0.26	1903	418
Normal plum[4]	75.1	6.72	694	59.3	3.12	0.11	183	24.0	-	-	-	-	72.9	35.2	-	-	1029	73.3
Präsident[4]	33.5	11.8	377	11.0	4.29	0.41	155	6.83	-	-	-	-	20.5	3.34	2.24	1.03	592	17.8
Cherry plum[4]	37.0	12.5	1838	279	10.6	0.48	16.1	1.77	-	-	-	-	52.0	11.8	2.80	0.00	1956	279

[*] All on wet weight basis

[1] n= 1 piece/variety

[2] n= 4-6 pieces/variety

[3] n= 2-3 pieces/variety

[4] n= 6-9 pieces/variety

[+] Varieties purchased/collected between September 2010 and November 2011 from a single location in Luxembourg

Table 3: Polyphenol content of the different *Brassica oleraceae* and plum varieties investigated.

Variety[+]	Chlorogenic acid		Neochlorogenic acid		Crypto-chlorogenic acid		Kaempferol-3-glucoside		Quercetin-3-*O* galactoside		(+)-Catechin		Gallic acid		Sinapic acid		p-Coumaric acid		Ferulic acid		4-Hydroxybenzoic acid		Total Polyphenols	
	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD	Mean µg/100 g	SD
Pointed cabbage[1]	–	–	3168	174	–	–	–	–	–	–	–	–	–	–	279	33.4	–	–	–	–	–	–	3446	177
White cabbage[1]	–	–	270	66.1	–	–	–	–	–	–	–	–	–	–	93.1	21.9	–	–	–	–	–	–	363	69.6
Kalorama[1]	–	–	51.1	7.86	–	–	–	–	–	–	–	–	30.1	5.94	59.7	13.2	–	–	–	–	–	–	141	16.4
Marner Lagerwhite 1[1]	–	–	87.6	6.53	–	–	–	–	–	–	–	–	–	–	69.5	1.87	–	–	–	–	–	–	157	6.79
Endive[1]	16,609	527	–	–	–	–	9185	1336	–	–	–	–	–	–	–	–	–	–	–	–	49.8	13.3	25,844	1436
German turnip[1]	–	–	–	–	–	–	–	–	–	–	–	–	737	17.1	–	–	–	–	–	–	–	–	737	17.1
Brussels sprouts[2]	–	–	4589	350	–	–	–	–	–	–	–	–	–	–	224	18.5	–	–	294	20.8	–	–	5107	352
Duchy[1]	–	–	2130	91.7	–	–	–	–	–	–	–	–	–	–	145	16.2	–	–	1014	34.0	–	–	3289	99.1
Toughma[1]	–	–	100	7.06	–	–	–	–	–	–	–	–	–	–	214	19.5	–	–	–	–	–	–	314	20.7
Cauliflower[3]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	508	56.9	–	–	–	–	130	4.13	638	57.0
Gigant DDR/ Superschmelz[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Lanro[1]	–	–	95.1	8.90	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	95	8.90
Marner Lagerwhite 2[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Dottenfelder[1]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Winterfürst[1]	–	–	583	101	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	583	101
Red cabbage[1]	–	–	1552	199	–	–	–	–	–	–	–	–	100	9.7	1495	105	–	–	–	–	–	–	3148	225
Subaro[1]	–	–	1086	132	502	58.2	–	–	–	–	–	–	–	–	636	73.6	–	–	–	–	–	–	2224	162
Reguma[1]	–	–	1288	77.3	536	26.7	–	–	–	–	–	–	–	–	876	75.9	–	–	–	–	–	–	2701	112
Marner Lagerred 1[1]	–	–	3470	467	791	112	–	–	–	–	–	–	–	–	397	43.8	–	–	–	–	–	–	4658	482
Radicchio[1]	35,772	2728	73.1	19.1	–	–	476	195	1106	293	–	–	532	176	2382	612	–	–	774	153	–	–	41,115	2828
Marner Lagerred 2[1]	–	–	1587	141	848	81.7	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2435	163
Granat[1]	–	–	3451	250	1375	209	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4826	326
Savoy cabbage[1]	–	–	2771	151	1133	41.6	–	–	–	–	–	–	–	–	29.8	6.94	–	–	–	–	–	–	3934	157
Scots Kale[1]	–	–	5468	1083	1367	258	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6835	1113
Kale[1]	5378	284	7064	325	1668	76.0	–	–	–	–	–	–	–	–	2804	147	–	–	10,086	787	–	–	26,999	913
Brokkoli Verde[3]	641	159	5614	200	–	–	–	–	–	–	–	–	–	–	394	31.7	–	–	–	–	–	–	6649	258
Brokkoli Marathon[3]	295	139	3904	228	–	–	–	–	–	–	–	–	–	–	150	32.6	–	–	–	–	–	–	4348	269
Centenar 1[4]	37,068	5234	37,971	5438	–	–	–	–	1163	67.2	425	42.4	309	25.4	–	–	–	–	–	–	–	–	76,936	7548
Ersinger[4]	–	–	23,185	1976	–	–	–	–	1545	74.1	430	6.82	271	10.2	–	–	–	–	–	–	–	–	25,431	1977
Orthenauer[4]	–	–	25,842	1136	826	42.3	–	–	1897	193	439	28.2	332	26.7	–	–	–	–	–	–	–	–	29,337	1153
Centenar 2[4]	16,086	661	29,299	1956	–	–	–	–	1744	132	308	7.84	–	–	–	–	–	–	–	–	–	–	47,437	2069
Duederer[4]	12,664	598	17,868	324	–	–	–	–	822	126	303	11.9	–	–	–	–	–	–	–	–	–	–	31,657	692
Green Gage plum[4]	9104	130	48,584	3335	1075	260	–	–	388	121	399	145	–	–	–	–	–	–	–	–	–	–	59,551	3353
Plum 620[4]	38,235	4731	81,685	3602	1537	463	–	–	1755	625	211	39.3	–	–	–	–	–	–	–	–	–	–	123,424	5997
Plum 605[4]	12,134	1315	33,432	2030	–	–	–	–	1056	215	1218	84.8	–	–	–	–	–	–	–	–	–	–	47,839	2430
Kuhpanz[4]	19,050	2751	32,514	2602	–	–	–	–	1151	179	188	9.81	–	–	–	–	–	–	–	–	–	–	52,903	3791
Plum 575[4]	9483	856	30,059	1658	–	–	–	–	619	89.8	111	10.6	–	–	–	–	–	–	–	–	–	–	40,271	1868
Mirabelle plum[4]	34,603	3909	40,140	1971	1780	133	–	–	1780	434	207	12.0	–	–	–	–	–	–	–	–	–	–	78,510	4401
Graf Althanns[4]	35,234	3286	51,739	4794	1466	263	90.6	15.2	1399	173	262	13.8	824	79.2	–	–	–	–	–	–	–	–	91,016	5821
Kirks plum[4]	30,400	2993	114,447	13,604	2608	395	104	2.88	3699	1543	466	125	–	–	–	–	182	131	66.5	15.4	–	–	151,973	14,021
Italian plum[4]	18,461	2377	62,031	6725	–	–	–	–	1985	216	288	23.4	–	–	–	–	–	–	–	–	–	–	82,766	7136
Normal plum[4]	8228	242	13,947	1946	–	–	–	–	677	190	69.9	19.4	682	139	–	–	–	–	–	–	–	–	23,604	1975
Präsident[4]	17,175	1577	61,439	2189	–	–	–	–	1845	355	182	25.2	–	–	–	–	–	–	–	–	–	–	80,461	2721
Cherry plum[4]	4643	788	6033	574	–	–	105	75.4	2781	792	138	63.9	754	126	–	–	–	–	–	–	–	–	14,454	1267

[1] n= 1 piece/Variety
[2] n= 4-6 pieces/Variety
[3] n= 2-3 pieces/Variety
[4] n= 6-9 pieces/Variety
[+] Varieties purchased/collected between September 2010 and November 2011 from a single location in Luxembourg

Table 4: Total phenolics, total flavonoids, total anthocyanins and related antioxidant capacity (ABTS, FRAP) of the different *Brassica oleraceae* and plum varieties investigated.

Variety[+]	Total phenolics[a]		Total flavonoids[b]		Total anthocyanins[c]		ABTS[d]		FRAP[e]		Vitamin C[f]		Selenium[g]	
	Mean mg/100g	SD	Mean mg/100g	SD	Mean mg/100g	SD	Mean mg/100g	SD	Mean µmol/100g	SD	Mean mg/100g	SD	Mean µg/100g	SD
Pointed cabbage[1]	12.5	1.16	8.04	0.39	0.14	0.11	87.5	3.52	314	12.8	65.0	2.03	0.59	0.03
White cabbage[1]	18.8	2.87	6.34	5.18	0.25	0.07	67.7	11.2	214	44.9	55.8	3.15	0.54	0.02
Kalorama[1]	5.77	1.62	0.96	0.16	0.11	0.09	28.9	4.72	164	17.1	36.0	5.78	0.29	0.02
Marner Lagerwhite 1[1]	8.88	1.20	2.23	0.87	0.52	0.36	42.4	3.69	207	12.3	45.1	1.48	0.48	0.02
Endive[1]	11.5	1.17	15.8	4.41	0.16	0.04	111	13.7	163	30.1	70.7	10.3	0.18	0.03
German turnip[1]	5.44	0.11	1.35	0.60	1.01	0.30	43.4	3.61	532	28.7	54.3	9.10	0.53	0.02
Brussels sprouts[2]	23.1	10.4	13.6	1.68	0.74	0.74	191	33.8	555	51.9	96.5	7.08	1.95	0.18
Duchy[1]	61.5	12.5	27.1	5.76	0.77	0.41	166	9.71	439	64.0	77.2	4.86	0.75	0.22
Toughma[1]	13.4	0.42	4.75	0.32	2.51	0.14	46.3	0.91	193	10.2	46.3	9.17	0.11	0.01
Cauliflower[3]	45.2	5.27	14.7	4.37	0.75	0.26	72.4	3.04	533	36.9	63.0	6.42	0.48	0.29
Gigant DDR/ Superschmelz[1]	15.8	0.69	0.76	0.07	1.78	0.39	58.2	1.85	231	26.2	40.1	7.42	0.20	0.03
Lanro[1]	11.2	1.94	5.65	1.24	0.87	0.66	38.3	1.88	177	18.6	33.3	7.63	2.06	0.67
Marner Lagerwhite 2[1]	22.6	3.98	2.14	0.23	6.46	0.97	81.1	19.4	335	13.1	33.6	9.54	0.13	0.01
Dottenfelder[1]	18.1	1.87	1.74	0.31	4.18	1.83	43.0	3.02	263	27.4	36.2	7.88	0.10	0.00
Winterfürst[1]	18.1	3.62	2.17	0.32	1.35	0.80	53.4	6.74	239	34.3	12.9	10.3	1.08	0.21
Red cabbage[1]	89.0	28.8	50.5	8.13	71.0	14.0	334	18.8	1656	284	176	4.91	0.53	0.02
Subaro[1]	103	15.8	32.8	4.37	54.2	12.1	178	17.1	882	79.2	74.9	1.56	0.33	0.02
Reguma[1]	98.1	4.18	29.2	0.92	61.7	12.3	227	33.2	906	59.5	111	11.3	0.36	0.00
Marner Lagerred 1[1]	87.3	16.8	27.8	2.86	21.8	5.49	161	6.25	844	5.45	105	0.52	0.31	0.00
Radicchio[1]	34.6	15.4	45.2	11.4	36.9	9.83	182	57.8	685	243	55.9	15.5	1.82	0.10
Marner Lagerred 2[1]	88.5	3.27	18.7	0.64	49.3	3.66	99.9	3.46	897	45.6	26.1	14.1	0.13	0.01
Granat[1]	139	22.7	41.3	6.48	35.8	5.54	382	55.4	1759	163	113	20.1	0.82	0.65
Savoy cabbage[1]	13.4	2.25	5.50	0.38	0.71	0.34	54.3	8.43	255	12.3	35.9	2.05	0.41	0.02
Scots Kale[1]	41.7	15.5	12.5	2.47	1.28	0.19	190	15.1	367	47.0	56.6	1.06	0.67	0.04
Kale[1]	60.2	4.49	50.0	1.08	0.29	0.29	381	4.10	953	249	75.9	16.2	0.95	0.08
Brokkoli Verde[3]	101	7.23	65.0	8.19	1.49	0.37	268	29.7	583	89.2	29.2	10.0	1.21	0.05
Brokkoli Marathon[3]	58.5	3.90	37.6	11.1	1.59	0.35	172	17.0	420	53.7	40.7	14.3	0.23	0.11

Table 4: Continued

Variety[+]	Total phenolics[a]		Total flavonoids[b]		Total anthocyanins[c]		ABTS[d]		FRAP[e]		Vitamin C[f]		Selenium[g]	
	Mean mg/100g	SD	Mean mg/100g	SD	Mean mg/100g	SD	Mean mg/100g	SD	Mean µmol/100g	SD	Mean mg/100g	SD	Mean µg/100g	SD
Centenar 1[4]	104	18.6	96.3	14.9	26.0	2.64	268	1.34	1759	66.2	43.3	7.42	0.63	0.02
Ersinger[4]	103	10.5	41.9	2.43	10.5	1.22	270	2.00	913	49.7	41.5	7.35	0.89	0.38
Orthenauer[4]	81.9	6.53	38.4	2.30	7.33	0.23	231	4.02	587	56.5	22.0	5.61	0.55	0.01
Centenar 2[4]	92.0	1.68	40.1	1.50	21.1	1.93	222	1.59	740	90.0	17.2	2.36	0.49	0.00
Duederer[4]	144	2.85	89.2	0.12	0.00	0.00	291	24.5	1648	243	25.1	5.87	0.68	0.01
Green Gage plum[4]	200	7.11	144	9.37	0.34	0.59	386	2.47	1782	527	22.0	4.44	0.86	0.01
Plum 620[4]	86.8	16.3	117	16.4	10.8	2.70	233	9.70	1451	427	8.69	1.75	0.57	0.01
Plum 605[4]	190	10.6	141	10.4	0.03	0.06	341	6.05	2919	466	16.1	4.56	1.05	0.44
Kuhpanz[4]	209	11.5	136	8.92	12.7	3.26	382	5.20	2437	408	19.7	4.82	0.81	0.02
Plum 575[4]	144	15.5	120	6.71	0.16	0.07	267	4.10	2155	81.5	14.0	4.76	0.79	0.30
Mirabelle plum[4]	138	18.5	65.3	7.39	0.71	0.39	351	21.7	1391	65.6	24.3	5.19	0.74	0.03
Graf Althanns[4]	117	9.64	77.5	0.14	3.37	0.47	195	1.86	1117	121	15.1	3.59	0.45	0.01
Kirks plum[4]	111	12.8	68.0	5.60	14.2	2.07	216	10.4	1148	26.1	4.14	0.41	0.60	0.15
Italian plum[4]	170	12.2	76.5	3.40	20.9	2.10	328	5.67	2264	126	24.6	3.06	0.73	0.05
Normal plum[4]	61.9	9.94	24.6	4.71	10.8	2.92	299	9.99	718	126	25.1	8.13	0.67	0.00
Präsident[4]	115	5.59	49.0	1.43	13.7	2.32	244	10.4	1191	1191	22.8	2.48	0.61	0.10
Cherry plum[4]	112	16.5	27.2	4.23	68.7	18.3	230	14.6	1687	1687	48.8	2.06	0.53	0.01

[a] Total phenolic content was expressed as mg (gallic acid equivalents)/100g fresh material. Data was obtained from three independent extractions.

[b] Total flavonoid content was expressed as mg (catechin equivalents)/100g fresh material. Data was obtained from three independent extractions.

[c] Total anthocyanin content was expressed as mg (cyanidin-3-glucoside)/100g fresh material. Data was obtained from three independent extractions.

[d] ABTS means are expressed as mg (ascorbic acid)/100g fresh material. Data was obtained from three independent extractions.

[e] FRAP means are expressed as µmol (FeII)/100g fresh materiel. Data was obtained from three independent extractions.

[f] Free vitamin C content. Data was obtained from three independent extractions

[g]Selenium content. Data was obtained from three independent extractions.

[1] n= 1 piece/Variety

[2] n= 4-6 pieces/Variety

[3] n= 2-3 pieces/Variety

[4] n= 6-9 pieces/Variety

[+] Varieties purchased/collected between September 2010 and November 2011 from a single location in Luxembourg

Table 5
Sugar content* of the different plum varieties as estimated by refractometry.

Variety	Sugar content	
	Mean g/100 g	SD g/100 g
Centenar	16.7	0.40
Ersinger	12.2	2.38
Orthenauer	13.0	0.15
Centenar	10.5	1.00
Duederer	11.4	3.38
Green Gage plum	18.6	1.97
Plum 620	12.6	0.96
Plum 605	17.9	2.61
Kuhpanz	19.6	1.07
Plum 575	11.1	2.86
Mirabelle plum	14.7	1.01
Graf Althanns	8.51	0.39
Kirks plum	12.5	0.36
Italian plum	12.1	4.14
Normal plum	12.7	3.44
Präsident	9.44	1.66
Cherry plum	8.97	1.24

$n = 6–9$ pieces of fruits/variety, SD based on triplicate measurements.
* All on wet weight basis.

4.4 Discussion

In the present study, we investigated the patterns of major phytochemicals, selected macro- and micronutrients and their related antioxidant capacity in various *Brassica* and plum varieties grown in Luxembourg, two major contributing vegetable and fruit groups to both carotenoid and polyphenol intake. The results highlight the large variations that exist in the genera of *Brassica* and *Prunus* with respect to nutrient and non-nutrient composition, and their association with total antioxidant capacity, emphasizing that phenolic constituents such as neochlorogenic acid (but also flavonoids and anthocyanins), ascorbic acid, but also the carotenoid lutein in *Brassica*, and total

sugars, selenium and the polyphenol chlorogenic acid in plums could reasonably predict antioxidant capacity as measured by ABTS and FRAP, indicating the importance of additive and/or synergistic effects contributing to antioxidant attributes in these plant foods. Furthermore, based on local food consumption data, it could be estimated that the consumption of *Brassica* and plum varieties contributed to an antioxidant intake equivalent to 26 and 6 mg of ascorbic acid equivalents, respectively.

In our study, plums and *Brassica* were chosen, as they represented the 4th most frequently consumed fruit and the 7th most frequently consumed vegetable type (Biehler *et al.*, 2012). The determined carotenoid profile was considerably different between the two plant food groups. In both plum and *Brassica* varieties, lutein and beta-carotene represented the most predominant carotenoids, however we found different profiles of the less abundant carotenoids. While *Brassica* varieties also contained detectable amounts of violaxanthin and neoxanthin but neither alpha-carotene nor phytoene, we detected alpha-carotene and phytoene but neither violaxanthin nor neoxanthin in plums. It is known that beta-carotene, lutein, violaxanthin and neoxanthin accumulate in green plant tissues in conjunction with chloroplasts (Engelmann, Clinton, & Erdman, 2011), while alpha-carotene is mostly found in yellow/orange fruits and vegetables, being in line with its detection in plum but not in *Brassica* varieties. Phytoene was only detected in some but not all plum varieties, and this precursor for other carotenoids was possibly completely converted into the observed carotenoids, either due to genetic reasons or their state of maturity. The strongest antioxidants, at least *in vitro*, are

lycopene > alpha-carotene > beta-cryptoxanthin > beta-carotene > lutein (Stahl *et al.*, 1998). Thus, varieties rich in the first carotenoids may correlate with higher antioxidant values in *in vitro* tests. However, this was not observed in our tests, possibly due to confounding polyphenols or vitamin C, also acting as antioxidants, and the limited solubility of carotenoids in the FRAP and ABTS test. *In vivo*, these carotenoids however are typically poorly absorbed (Bohn, 2008), and may be cleaved further by beta-carotene mono or diooxygenases, thus their behaviour *in vivo* is difficult to predict. In the present study, total carotenoids were poorly correlated with the antioxidant capacity in plums as opposed to the more water soluble polyphenols. It is further possible that the lower concentrations of carotenoids in plums (Table 2) contributed to this observation, especially considering that in *Brassica*, at least for the FRAP assay, lutein, the most predominant and least hydrophobic carotenoid in *Brassica*, constituted a suitable copredictor of antioxidant capacity. The FRAP assay is based on electron transfer (Prior, Wu, & Schaich, 2005), and as carotenoids may act as electron donors or acceptors (Martin *et al.*, 1999), the results are in line with our finding as constituting a better predictor for FRAP than for the ABTS assay, which is based on hydrogen transfer (Prior *et al.*, 2005).

Concerning individual polyphenols in the *Brassica* and plum varieties, chlorogenic acid and its isomers, neo- and cryptochlorogenic acids were the most predominant ones in both groups, and both chlorogenic acid as well as its isomer neochlorogenic acid were important predictors for antioxidative capacity in plum and *Brassica* varieties, respectively. Other individual polyphenols such as the

hydrocinnamic acids (ferulic, p-coumaric and sinapic acid), phenolic acids (gallic acid and 4-hydroxybenzoic acid) and glycosides of flavonols (quercetin-3-O-galactoside and kaempferol 3-O-glucoside) contributed only little to the total polyphenol content (<25%). The flavonol catechin and the two glycosides of flavonols kaempferol and quercetin were only detected in plum varieties and in the cichorium varieties, while sinapic acid was only detected in *Brassica*. The total phenolic content as determined by Folin–Ciocalteu was well correlated with ABTS and FRAP, both in *Brassica* and plum. However, the sum of the individual polyphenols as detected by UPLC showed a good correlation with antioxidant capacity only in *Brassicae* (ABTS, $R = 0.855$, $P < 0.01$) but not in plums, indicating, as remarked in previous studies, that Folin–Ciocalteu is not selective for polyphenols and may considerably overestimate the contribution of polyphenols to antioxidant capacity.

While the total phenolic and flavonoid content as well as the antioxidant capacity were higher in plum varieties, the ascorbic acid content was higher in cabbage varieties, perhaps reflected by constituting a predictive factor in the multiple regression model. Ascorbic acid can be classified as a potent water-soluble antioxidant, and acts preferably via hydrogen donation, which may explain why it was a stronger predictor for the ABTS than the FRAP test, and several *in vitro* studies have shown that ascorbic acid can reduce LDL oxidation (Jialal & Fuller, 1995). In addition to ascorbic acid, the trace element Se correlated well with ABTS, at least in plums, and was an important predictor for antioxidant capacity, both for FRAP and ABTS. Selenium is an important component of antioxidant enzymes

such as glutathione peroxidase and thioredoxin reductase (Flohé & Brigelius-Flohé, 2012), which are important for maintaining the cellular redox balance, but it is not clear why it was a good predictor for antioxidant activity in plums in the present study. It cannot be excluded that it merely indicated higher dry mass content to which it was correlated, thus indicating a higher concentration of various antioxidants.

As a limitation it should finally be stated that the present study would not allow to draw conclusions on constituents depending on individual cabbage and plum varieties, as for this, collection of several specimens from various regions and from several years would have been required. Thus, the study rather presents an estimation of the scope of the varying nutrients and non-nutrients in *Brassica* and plum varieties, and their relation to antioxidant activity.

However, all individual components of the exogenous antioxidant system have to be derived from the diet, and thus dietary intake as well as their absorption play an important role. In Luxembourg, the consumption of *Brassica* and plums is relatively high, with 6.8 and 0.6 kg/year and person, respectively. This is, for vegetables the 7th important source after e.g. potatoes (24 kg/year and person) and tomatoes, and for plums the 4th most important source, after e.g. apples (22 kg/year and person) and citrus fruits (Biehler *et al.*, 2012). Although their total contribution to daily carotenoid and polyphenol content was comparably small, around 2–4% of daily intake for carotenoids and less for the polyphenols, several of the *Brassica* and plum varieties such as Kale and Cherry plum possessed rather high concentrations of ascorbic acid, total carotenoids and total

polyphenols and could be considered as good contributors to the intake of antioxidant active constituents.

4.5 Conclusion

In summary, the present study shows the range of nutrients and phytochemicals within various *Brassica oleraceae* and plum varieties and highlights the role of the individual ingredients in relation to different measures of antioxidant capacity, showing that for *Brassica* phenolic constituents, vitamin C and even lutein were the best predictors for antioxidant capacity, and selenium, sugars, and especially the polyphenol chlorogenic acid for plums.

4.6 Supplemental Data

Table 1: Micro- and Macronutrients of our different cabbage and plum varieties.

Variety[+]	Mineral Concentrations					
	Total Quantity elements [a]		Total essential Trace elements [b]		Total Heavy Metals [c]	
	Mean mg/100g	SD	Mean mg/100g	SD	Mean mg/100g	SD
Pointed cabbage[1]	512	11.8	0.70	0.03	0.02	0.00
White cabbage[1]	586	2.51	1.12	0.67	0.05	0.00
Kalorama[1]	244	4.35	0.32	0.02	0.02	0.00
Marner Lagerwhite 1[1]	308	3.09	0.36	0.01	0.02	0.00
Endive[1]	178	1.72	0.18	0.01	0.02	0.01
German turnip[1]	328	5.84	0.43	0.01	0.03	0.00
Brussels sprouts[2]	802	13.2	9.16	0.14	0.17	0.00
Duchy[1]	569	13.7	1.01	0.11	0.04	0.00
Toughma[1]	264	8.99	0.29	0.02	0.02	0.00
Cauliflower[3]	501	78.7	0.89	0.29	0.03	0.00
Gigant DDR Superschmelz[1]	446	23.9	0.30	0.04	0.01	0.00
Lanro[1]	346	7.44	0.57	0.08	0.01	0.00
Marner Lagerwhite 2[1]	378	12.8	0.37	0.02	0.02	0.00
Dottenfelder[1]	355	28.1	0.35	0.06	0.03	0.00
Winterfürst[1]	429	17.1	0.84	0.27	0.04	0.01
Red cabbage[1]	414	4.59	0.41	0.01	0.02	0.00
Subaro[1]	283	5.13	0.40	0.01	0.02	0.00
Reguma[1]	286	3.54	0.45	0.00	0.03	0.00
Marner Lagerred 1[1]	261	2.21	0.31	0.01	0.03	0.00
Radicchio[1]	342	15.1	2.01	0.11	0.02	0.00
Marner Lagerred 2[1]	396	2.86	0.40	0.00	0.02	0.00
Granat[1]	330	30.0	0.51	0.23	0.02	0.00
Savoy cabbage[1]	358	5.81	0.60	0.03	0.05	0.00
Scots Kale[1]	466	8.53	0.49	0.02	0.04	0.00
Kale[1]	1033	24.4	1.16	0.01	0.14	0.00
Brokkoli Verde[3]	537	9.74	1.83	0.03	0.07	0.00
Brokkoli Marathon[3]	413	5.00	0.72	0.02	0.05	0.00
Centenar 1[4]	218	50.5	0.15	0.03	0.06	0.01
Ersinger[4]	246	7.97	0.13	0.01	0.06	0.00
Orthenauer[4]	218	6.02	0.10	0.00	0.05	0.00
Centenar 2[4]	217	3.62	0.11	0.01	0.03	0.00
Duederer[4]	239	2.31	0.10	0.01	0.06	0.00
Green Gage plum[4]	368	6.86	0.14	0.01	0.11	0.01
Plum 620[4]	224	3.01	0.19	0.04	0.04	0.00
Plum 605[4]	256	9.96	0.17	0.03	0.03	0.00
Kuhpanz[4]	242	5.61	0.29	0.01	0.04	0.00
Plum 575[4]	223	4.17	0.12	0.02	0.06	0.01

Mirabelle plum[4]	263	5.94	0.15	0.00	0.06	0.00
Graf Althanns[4]	214	1.01	0.14	0.00	0.06	0.00
Kirks plum[4]	229	4.67	0.08	0.03	0.05	0.00
Italian plum[4]	280	9.11	0.15	0.01	0.08	0.00
Normal plum[4]	245	1.79	0.11	0.01	0.05	0.00
Präsident[4]	229	6.97	0.13	0.01	0.04	0.00
Cherry plum[4]	302	9.73	0.16	0.01	0.07	0.00

[a] Total Quantity elements = Na, Mg, K, Ca. Data was obtained from three independent extractions.
[b] Total essential Trace elements = Cr, Fe, Se. Data was obtained from three independent extractions.
[c] Total Heavy metals =Pb, Cd, Cu. Data was obtained from three independent extractions.
[1] n= 1 piece/Variety/ [2] n= 4-6 pieces/Variety/ [3] n= 2-3 pieces/Variety/ [4] n= 6-9 pieces/Variety/
[+] Varieties purchased/collected between September 2010 and November 2011 from a single location in Luxembourg

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Chapter 5: Carotenoid and Polyphenol Bioaccessibility and Cellular Uptake from Plum and Cabbage Varieties

In this chapter 5, we investigated how bioaccessible the carotenoids and polyphenols were, as well as their cellular uptake, with the help of in-vitro gastro intestinal digestion and a Caco-2 monoculture or Caco-2/HT-29 MTX coculture cell model. The results have resulted in an article with the title: Carotenoid and Polyphenol Bioaccessibility and Cellular Uptake from Plum and Cabbage Varieties, Kaulmann, A., André, C.M., Schneider, Y.J., Hoffmann, L., Bohn, T., Food Chem., Volume 197, Part A, 15 April 2016, pp. 325-332.

Carotenoid and Polyphenol Bioaccessibility and Cellular Uptake from Plum and Cabbage Varieties

Abstract

Plum and cabbage are rich in carotenoids and polyphenols. However, their bioactivity depends on their release and intestinal uptake. Four varieties of *Brassicaceae* (Duchy, Scots Kale, Kalorama) and *Prunus* (Cherry Plum, Plum 620, Ersinger, Italian Plum) were studied; bioaccessibility following *in vitro* digestion, cellular uptake (Caco-2 vs. co-culture cell model: Caco-2:HT-29-MTX (90:10%) and colonic fermentation were determined for carotenoids/polyphenols; the influence of certain kitchen preparations was likewise studied. Carotenoids were non-significantly influenced by the latter, while for polyphenols, boiling and steaming significantly reduced total phenolics ($p < 0.05$). Carotenoid bioaccessibility did not differ significantly between *Prunus* vs. *Brassicaceae* varieties, but xanthophyll was higher than carotene bioaccessibility ($p < 0.01$). Polyphenol bioaccessibility was low (<10%), possibly compromised by the cream containing test meal. Total carotenoid cellular uptake varied between varieties (0.3–4.1%), being higher for carotenes (4.1%) than for xanthophylls (1.6%, $p < 0.01$), and were higher for the co-culture cell model compared to Caco-2 cells ($p < 0.01$). Total carotenoid recovery in the colonic fraction varied from 4% to 25%. Lower bioaccessibility of carotenes thus appeared to be somewhat counterbalanced by higher cellular uptake. The potential positive role of the mucus layer for cellular uptake and the fate of the colonic digesta deserve further attention in the future.

5.1 Introduction

Diets rich in fruits and vegetables are recommended by many health organizations, and epidemiological studies have shown that their consumption can decrease the incidence of several chronic diseases such as type II diabetes (Carter, Gray, Troughton, Khunti, & Davies, 2010), cancer, and cardiovascular diseases (Dauchet, Amouyel, Hercberg, & Dallongeville, 2006). Fruits and vegetables contain an array of phytochemicals, and until today it is unknown which constituents are the most bioactive with respect to beneficial health effects.

Carotenoids and polyphenols represent the most abundant lipid and water soluble phytochemicals, respectively. Cabbage and plum varieties contain reasonable high concentrations of carotenoids/ polyphenols, up to ca. 60/40 mg per 100 g for cabbages and 2/125 mg per 100 g for plums, respectively (Kaulmann, Jonville, Schneider, Hoffmann, & Bohn, 2014), and are thus valuable sources of secondary plant compounds. In Luxembourg, for example, *Brassica oleracea* and *Prunus* consumption amounts to 14 kg/year and 16 kg/year, respectively.

Upon ingestion, the bioaccessibility of carotenoids and polyphenols depends on several factors (Bohn, 2008; Bohn, 2014). Carotenoids, with lutein and beta-carotene being the most prominent ones in plums and cabbages, need to be released mechanically and/or enzymatically from the food matrix and (due to their hydrophobicity) require, for solubilisation, incorporation into mixed micelles before they can be taken up by small intestinal enterocytes (Sy *et al.*, 2012).

As carotenoids in fruits and vegetables are often located in chromoplast (or chloroplast) organelles, their sub-structure and the cell wall are the main barriers for carotenoid bioaccessibility (Palmero, Panozzo, Simatupang, Hendrickx, & Van Loey, 2014). Thermal processes such as boiling or steaming can have positive effects on bioaccessibility, by helping to disrupt the food matrix, though negative influences due to degradation of carotenoids, especially once released from the matrix, have also been reported (Palmero *et al.*, 2014). Following matrix release and transfer into mixed micelles, the next barrier for carotenoid absorption represents the mucus layer in the intestine. A study by Meaney and O'Driscoll (1999) showed that the mucus layer can have a negative impact on the availability of apolar compounds such as of carotenoids.

The bioaccessibility of polyphenols appears more complex, due to the many different polyphenols existing, their changes during digestion and metabolism, depending largely on their polarity, molecular weight, glycosylation and esterification. Polyphenols first need to be released from the food matrix, taking place during the gastric phase (Bouayed, Hoffmann, & Bohn, 2012), and the majority of rather polar polyphenols are available for absorption without micelle incorporation; however, further cleavage into the respective aglycon may be required. Apparent polyphenol concentrations may be increased following digestion, possibly as non-extractable polyphenols (NEPP) are released (Saura-Calixto, Serrano, & Goni, 2007). The same may be true for kitchen preparation procedures, although many household procedures may activate enzymes such as

phenol-oxidase, thereby degrading polyphenols (Terefe, Delon, Buckow, & Versteeg, 2015). However, most cabbage varieties are not consumed raw but cooked, and treatments including chopping, freezing, thawing, in combination with different heat treatments, can be expected to influence the concentration of bioactive compounds.

The aim of the present study was to study the bioaccessibility of carotenoids and polyphenols from selected plum and cabbage varieties following simulated gastrointestinal digestion. Furthermore, cellular uptake of carotenoids and polyphenols post-digestion was investigated by Caco-2 and Caco-2/HT-29-MTX cell models, to examine the influence of the mucus layer. Finally, a colonic fermentation step was included to study further degradation pathways of carotenoids. Common kitchen preparation procedures were additionally employed to exemplary determine their influence on carotenoid and polyphenol content of cabbage.

5.2 Material and methods

5.2.1 Chemicals and standards

All products were of analytical grade or higher. MilliQ water was prepared with a purification system from Millipore (Brussels, Belgium) and was used for all experiments. Unless otherwise stated, all chemicals including methyl-tert-butyl ether (MTBE), porcine pepsin (P250 U/mg solid, measured as TCA-soluble products using hemoglobin as substrate), porcine bile mixture, and porcine pancreatin (4X USP specifications) were procured from Sigma–Aldrich (St. Louis, MO, USA). Acetone and sodium chloride were purchased from VWR (Haasrode, Belgium). Ammonium acetate,

methanol, hexane, acetonitrile and dichloromethane were obtained from BioSolve (Valkerswaard, the Netherlands). Phosphate buffer solution pH7 was procured from Chemlab (Zedelgem, Belgium). Carotenoid standards, *i.e*. β-carotene, β-cryptoxanthin and lutein were from Extrasynthèse (Lyon, France), while α-carotene, violaxanthin, neoxanthin and phytoene were obtained from CaroteNature (Lupsingen, Switzerland), and trans-β-apo-8'-c arotenal (internal standard (IS) to check for chromatographic recovery of carotenoids) was from Sigma–Aldrich. Certified purity of all standards was above 95%. Polyphenol standards, *i.e*. (+)-catechin, caffeic acid, *p*-coumaric acid, cryptochlorogenic acid (4-caffeoylquinic acid), neochlorogenic acid (3-caffeoylquinic acid), quercetin, quercetin 3-O-galactoside, kaempferol, kaempferol 3-O-glucoside, ferulic acid, syringic acid, sinapic acid, gallic acid and vanillic acid were obtained from Sigma–Aldrich. Chlorogenic acid and cinnamic acid were purchased from Merck (Darmstadt, Germany). Kaempferol-3-O-rutinoside was obtained from Extrasynthèse and 4-hydroxybenzoic acid was purchased from Thermo Fisher Scientific (Geel, Belgium). Cell media (DMEM +GlutaMAX), PBS, and FBS were obtained from GIBCO (Karlsruhe, Germany).

5.2.2 Plum and cabbage varieties

Plums and cabbages were procured and characterized for micro- and macro-constituents as described previously (Kaulmann *et al.*, 2014). In brief, 27 cabbage and 17 plum varieties were obtained from Luxembourgish supermarkets or farmers. Varieties rich in either carotenoids or polyphenols, both, or being low in carotenoids or polyphenols or both were finally retained for further analyses:

Plums (Cherry Plum, Plum 620, Ersinger, Italian Plum) and *Brassicaceae* varieties (Duchy, Scots Kale, Kale, Kalorama), chosen due to their contrasting content of carotenoids and polyphenols (Table 1).

All plum and *Brassicaceae* varieties were lyophilized (Christ freeze dryer, Thermo Fisher Scientific, Geel, Belgium) during 24 h, homogenized with a grinder (Motor Grinder RM 200, Retsch, Aarteselaar, Belgium) and stored in 50 mL plastic centrifuge tubes at -20 °C until analysis.

5.2.3 Kitchen procedures

As most vegetables are typically not consumed in raw form, different cooking procedures were investigated. Kale was used as a reference model to investigate the effect of freezing and thawing alone as well as the effect of chopping combined with boiling or steaming or freezing/thawing. To evaluate the effect of freezing and thawing, 50 g of kale leaves were divided into two equal parts (25 g). One part (denoted as control) was directly crushed under liquid nitrogen to powder, transferred into a 50 mL falcon tube and stored at -80 °C. The other part was first frozen for 48 h at -20 °C and then thawed for 4 h (denoted as freeze–thaw) before it was crushed under liquid nitrogen to powder and stored at -80 °C until analysis.

To evaluate the effect of chopping and boiling or steaming, 360 g of kale leaves were divided into four equal parts. One part (denoted as control) was first chopped into equal pieces of ca. 50 cm^2 with a kitchen knife, and then directly crushed under liquid nitrogen to

powder and stored at -80 °C. A second aliquot (denoted as chop/boiling) was first chopped into equal pieces of 50 cm^2 and then boiled for 7 min in 500 mL hot tap water before the leaves were then wiped with paper towels, and then crushed under liquid nitrogen to a powder and stored at -80 °C. A third part (denoted as chop/steaming) was first chopped into equal pieces of 50 cm^2 and then steamed for 7 min in a household steamer (Tefal VS4003, Offenbach, Germany), before the leaves were then wiped with paper towels and crushed under liquid nitrogen to a powder and stored at -80 °C. A fourth part (described as chop/freeze–thaw) was first chopped into equal pieces of 50 cm^2, frozen for 48 h at -20 °C, and then thawed for 4 h, before it was crushed under liquid nitrogen to a powder and stored at -80 °C. Carotenoid extraction was described elsewhere (Kaulmann *et al.*, 2014). All experiments were done in triplicates.

Table 1
Native content of carotenoids and polyphenols and their bioaccessibility after simulated in vitro gastrointestinal digestion of different cabbage and plum varieties.

	Native content in mg/100 g FW		Bioaccessibility in % of native content			
	Total carotenoids*	Total polyphenols#	Total carotenoids ± (SEM)*	Total xanthophylls ± (SEM)	Total carotenes ± (SEM)	Total polyphenols ± (SEM)#
Duchy	8.1	3.3	6.6 (0.1)^{CD}	7.9 (0.1)^{D}	3.5 (0.1)^{B}	Nd
Kale	13	27	6.8 (0.9)^{CD}	7.4 (1.0)^{D}	4.1 (0.6)^{B}	6.3 (0.01)
Scots Kale	1.0	6.8	0.9 (0.2)^{A}	0.8 (0.2)^{A}	1.4 (0.1)^{A}	Nd
Kalorama	0.1	0.1	3.6 (0.5)^{B}	2.4 (0.2)^{B}	9.2 (1.8)^{C}	Nd
Mean cabbage	5.5	9.3	4.5 (1.1)^{BC,a}	4.6 (1.0)^{C,a}	4.5 (1.9)^{B,a}	6.3 (0.01)
Cherry Plum	2.0	15	3.6 (0.2)^{B}	5.0 (0.4)^{C}	1.4 (0.2)^{A}	Nd
Plum 620	0.6	123	11 (0.3)^{D}	15 (0.4)^{E}	9.4 (0.6)^{C}	0.14 (0.001)
Ersinger	0.5	25	9.0 (0.4)^{D}	14 (0.7)^{E}	4.0 (0.3)^{B}	0.38 (0.003)
Italian Plum	2.0	83	3.4 (0.3)^{B}	9.8 (0.7)^{D}	1.9 (0.3)^{A}	0.27 (0.003)
Mean plum	1.3	62	6.8 (0.6)^{CD,b}	10.9 (1.1)^{DE,c}	4.2 (0.8)^{B,a}	0.26 (0.004)

Each value presents the mean ± SEM of three independent replicates.
Mean values in a column sharing the same capital letter symbol are not significantly different. Mean total carotenoids, xanthophyll and carotene values in a row sharing the same small letter are not significantly different ($p < 0.05$). FW = fresh weight. Nd = not detectable.

* Total carotenoids as determined by UPLC were the sum of: α-carotene, β-carotene, lutein, violaxanthin, neoxanthin, β-cryptoxanthin, phytoene, phytofluene.

Total polyphenols as determined by UPLC were the sum of: chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, kaempferol-3-glucoside, quercetin-3-O-galactoside, catechin, gallic acid, sinapic acid, *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid.

5.2.4 Simulation of gastro-intestinal passage

The *in vitro* digestion protocol was adapted from an earlier study (Biehler, Kaulmann, Hoffmann, Krause, & Bohn, 2011). Briefly, 500 mg of dried cabbage or dried plum were weighted into a 50 mL tube and mixed with 3 g of cream containing 12% fat, also as described earlier, in order to simulate a complete test meal containing fat, and to assure sufficiently high micellarization of carotenoids requiring the presence of some lipids. Milk was chosen as some cabbage and plum dishes (e.g. frozen kale containing cream, plum-cake with cream) do occasionally contain dairy products. Then, 10 mL of 0.15 M NaCl was added, and the mixture was homogenized for 2 min with an Ultra-Turrax (T25 basic, IKA Werke, Staufen, Germany). Fifteen mL of 0.15 M NaCl were further added and the whole test meal was acidified with 0.2–0.5 mL of 1 M HCl to a pH of 3. Two mL of porcine pepsin (40 mg/mL in 0.1 M HCl) were then added and the samples were sealed with Parafilm M (Brand GmbH + CO KG, Wertheim, Germany). The samples were then placed into a closed plastic bag and transferred to a shaking water bath for 1 h (GFL 1083 from VEL, Leuven, Belgium; 100 rpm, 37 °C). The pH of the post-gastric meal was then increased to 5.5 with 0.5–0.6 mL of 0.9 M $NaHCO_3$. In order to mimic the duodenal digestion, 9 mL of a pancreatin (4 mg/mL in 0.1 M $NaHCO_3$) and porcine bile extract (24 mg/mL in 0.1 M $NaHCO_3$) mixture was added to the samples. The pH of the samples was further increased to 7 with 0.1–0.2 mL of 1 M NaOH. The volume of the samples was adjusted to 50 mL with 0.15 M NaCl. The 50-mL tubes were sealed with Parafilm and incubated in a shaking water bath (100 rpm, 37 °C) for two hours. Following

centrifugation (2 min, 1000*g*), the digesta were then stored at -80 °C until further experiments.

5.2.5 Cell culture and uptake experiments

The TC-7 subclone (ATCC Number: HTB-37) of the Caco-2 parental cell line was derived from a tumor isolated by J. Fogh (Chantret *et al.*, 1994) and was a generous gift from Monique Rous-set (Nancy University, France). The HT-29-MTX cell line (ATCC Number: HTB-38) was a kind gift from Dr. Técla Lesuffleur (Institut National de la Santé et de la Recherche Médicale, INSERM UMR S 938, Paris, France). Cultures were routinely maintained in 75 cm^2 plastic flasks (Nunclon™, Nunc, Denmark) at 37 °C and 10% CO2 in a CB-210 CO2 incubator (Binder GmbH, Tuttlingen, Germany). Cells were grown in Dulbecco's modified eagle medium (DMEM + GlutaMAX™, Gibco, Karlsruhe, Germany), supplemented with 1% nonessential amino acids, 10% heat-inactivated fetal bovine serum (Gibco, Karlsruhe, Germany), 1% of a mixture of penicillin and streptomycin (10,000 units and 10 mg per mL, respectively) and subcultured weekly after reaching ca. 80% confluence.

For the monoculture (Caco-2 cells) uptake experiments, cells at passage numbers 80 to 85 were seeded into 6 well-plates (BD Bioscience, Erembodegem, Belgium) at a cell density of 5 * 10^4 cells/cm^2, 15 days prior to experiments. For the co-culture (Caco-2/HT29-MTX cells) uptake experiments, cells (passage numbers of 86 to 93 for Caco-2 cells and 19 to 26 for HT29-MTX cells) were seeded into 6 well plates at a cell density of 5 *10^4 cells/cm^2, and at a cell ratio of 90:10 (Caco-2/HT-29-MTX). Repeated measurements over

several days did not show any significant differences in TEER (transepithelial electrical resistance mΩ/cm^2) values. For the uptake experiments, medium was removed and cells were washed with 2 mL of PBS (Gibco, Karlsruhe, Germany) before they were incubated for 4 h with 2 mL of medium diluted complete digesta (obtained after small intestinal digestion, centrifuged for 2 min at 1000*g*) (1:8 digesta:DMEM, v:v) at 37 °C and 10% CO_2 as described earlier (Biehler & Kaulmann, *et al.*, 2011). Cell studies were performed in triplicate and on three different days (n = 3; N = 3).

5.2.6 Colonic fermentation

For the preparation of inocula, the protocol was adapted from Bindelle *et al.* (2007). In short, mixed inocula were prepared from faeces of three different pigs, in order to reduce variation. These were placed in a plastic sealed syringe in a water-bath at 39 °C. Then, 17 g of each sample was placed into a plastic bag containing saturated CO2 and 180 mL of buffer solution (composed of salt and minerals) and subjected for 60 s to a mechanical shaking with a Stomacher Lab Blender 400 (Seward Medical, Norfolk, UK). The solution was then filtered through a 250 µM mesh screen and the volume was adjusted to 820 mL with buffer solution to reach a concentration of 5% (w/v) faeces. For *in vitro* fermentation, 10 mL of the previously prepared complete digesta (obtained after centrifugation of the small intestinal digesta at 2 min and 1000*g*) was then mixed with 20 mL of freshly prepared inoculum in a 100 mL glass bottle and incubated for 24 h at 39 °C.

5.2.7 Carotenoid and polyphenol extractions

Carotenoid extraction details from digesta and the cell culture are described elsewhere (Biehler & Kaulmann, *et al.*, 2011). For the colonic fermented samples, 5 mL of sample was transferred into a 50 mL centrifuge tube and 2.5 mL of saturated NaCl and 7 mL of hexane:acetone (1:1, v/v) were added. The mixture was shaken for 1 min and centrifuged for 2 min at 4000*g*. The supernatant was transferred into a 15 mL centrifuge tube. Then, 4 mL of pure hexane was added to the 50 mL centrifuge tube, shaken for 1 min and centrifuged for 2 min at 4000*g*. Again, the supernatant was transferred to the 15 mL centrifuge tube. The extraction process with hexane was repeated once. The combined supernatants were dried under a stream of nitrogen at 30 °C. For polyphenol detection, digested samples were not extracted in order to increase detectability. Prior to UPLC injection, 1 mL of sample was filtered through a 0.2 µm filter (PALL Life Sciences, Ann Arbor, MI, USA) into a UPLC vial. After the incubation of the cells with the diluted digesta, cells were washed with 2 mL of cold PBS, and then 500 µL of cold water:MeOH (1:1, v/v) mixture was added and cells were incubated during 20 min at room temperature. Cells of 6 wells were pooled to improve detection sensitivity. Samples were then sonicated for 5 min and centrifuged at 2000*g* for 10 min. The supernatant was then transferred into a new 15 mL centrifuge tube and the pellet was then re-extracted with 1 mL of 100% methanol, including a second sonication (5 min) and centrifugation step (10 min at 2000*g*). The supernatants were combined and dried under a stream of nitrogen at

40 °C. If the samples were not used directly, they were overlayered with a blanket of argon and stored at -80 °C.

5.2.8 Quantification of carotenoids and polyphenols

For the UPLC analyses of carotenoids, dried extracts were spiked with appropriate amounts of the IS, dissolved in 600 µL cold MTBE: methanol (1:1: v:v), and filtered through a 0.2 µm filter (PALL Life Sciences, Ann Arbor, MI, USA) into cold UPLC amber vials. For the UPLC analysis of polyphenols, extracts were filtered through a 0.2 µm filter (PALL Life Sciences, Ann Arbor, MI, USA) into a UPLC vial.

For carotenoid quantification, an Acquity UPLC BEH C18 column (Waters Inc., Zellik, Belgium, 2.1 x 100 mm, 1.7 µm particle size, set at 40 °C) was used in combination with a Waters Acquity UPLC® system (Milford, MA) equipped with a photodiode array detector. The eluents were (A) ammonium acetate (50 mM) in water: methanol (60:40%, v/v) and (B) acetonitrile: dichloromethane (80:20%, v/v) and the gradient was as follows: 0 min, 42% B, flow rate 0.35 mL/min; 4 min, 47% B; 13 min, 80% B, flow rate 0.40 mL/min; 18 min, 85% B; 29 min, 85% B, flow rate 0.35 mL/ min; 30 min 42% B. The injection volume was 2.5 lL. Carotenoids were detected at 286 nm (phytoene), 440 nm (neoxanthin, violaxanthin), 450 nm (lutein, b-carotene and a-carotene) or 455 nm (zeaxanthin and b-cryptoxanthin) according to their absorption maxima. For quantification, 7 point – linear calibration curves were prepared with external standards for each compound, with concentrations ranging from 0.01 to 25 µg/mL. In

addition, the internal standard was measured as an additional chromatographic control.

The detection of polyphenols was adapted from Deusser, Guignard, Hoffmann, and Evers (2012). For the quantification of polyphenols, a Waters Acquity UPLC® system equipped with a photodiode array detector was employed. For separation, an Acquity UPLC® HSS T3 column (1.8-lm particle size, 2.1 x 100 mm) with a flow rate of 0.75 mL/min at 50 °C was used. The eluents were 0.1% (v/v) formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the gradient was as follows: 0 min, 5% B; 9.27 min, 5% B; 13.53 min, 14% B; 22.60 min, 35% B; 23 min, 95% B; 25 min, 95% B; 26 min, 5% B. The injection volume was 10 lL. Polyphenols were detected at 254 nm, 280 nm, 300 nm or 320 nm, according to their absorption maxima. For quantification, 7 point – linear calibration curves were prepared with external standards for each compound, with concentrations ranging from 0.01 to 50 mg/L. Samples with higher concentrations were appropriately diluted with water/ methanol (90:10 v/v). The sum of polyphenols (UPLC) was detected as the sum of the 18 individual polyphenols.

Total phenolic content following kitchen preparation procedures was determined with Folin–Ciocalteu's phenol reagent as described by Kaulmann *et al.* (2014), based on spectrophotometric analysis (DU800 UV/Visible spectrophotometer, Beckman Coulter, Palo Alto, CA, USA).

5.2.9 Data interpretation

Unless otherwise stated, all data represent mean ± standard error of mean (SEM). All data was evaluated by SPSS vs 19.0 (IBM, Chicago, IL, USA). Normal distribution of results and equality of variance were verified by Q–Q plots and box plots, respectively. Data was log-transformed to achieve normal distribution. For predicting differences in carotenoid and polyphenol profiles following selected kitchen preparation procedures, differences in bioaccessibility of carotenoids and polyphenols after *in vitro* digestion, differences in the cellular uptake and following colonic fermentation of carotenoids and polyphenols between the different cell models, linear mixed models were developed with carotenoid or polyphenol content as the dependent variables and kitchen procedures, carotenoid type, family (cabbage, plum) and cabbage and plum varieties (nested within family as random factor) as independent variables. P-values below 0.05 (2-sided) were considered statistically significant. Significant Fisher-F-values were followed by either Bonferroni or LSD tests (for comparison of up to 3 groups). All recoveries given represent percentages of the respective native content.

5.3 Results

No significant differences were detected for carotenoids following different kitchen treatment procedures (Fig. 1). For polyphenols, freezing followed by thawing did not alter total phenolic content significantly, while chopping followed by boiling and chopping followed by steaming significantly decreased total polyphenol content by 89% and 51%, respectively ($p < 0.05$, Fig. 2).

The bioaccessibility of total carotenoids from cabbage and plum varieties differed significantly, and was highest in Kale and lowest in Scots Kale (Table 1). Similarly, Plum 620 showed highest and Italian Plum lowest bioaccessibility. Bioaccessibilty of total carotenoids from plum varieties vs. cabbage varieties did not differ significantly. Xanthophylls (the sum of lutein and beta-cryptoxanthin, when present) showed generally a significantly higher bioaccessibility than carotenes ($p < 0.001$) in the different varieties.

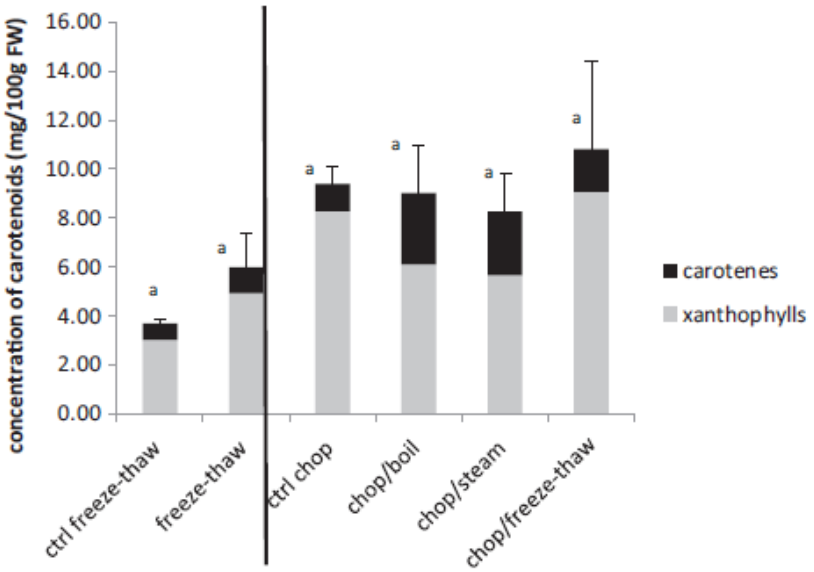

Fig. 1. Effect of different kitchen procedures on total carotenoid content. The effect of freezing (overnight, −20 °C) combined with thawing (freeze–thaw) was compared to kale crushed under liquid nitrogen and stored until analysis (−80 °C, ctrl freeze–thaw), and the effect of and chopping and boiling (chop/boil), chopping and steaming (chop/steam) as well as chopping and freezing and thawing (chop/freeze–thaw) vs. chopping only (kale cut into ca. 50 cm^2 pieces, than crushed under liquid nitrogen and stored at −80 °C until analysis, ctrl chop) on total carotenoid content was evaluated via UPLC detection. Each bar represents the mean total carotenoids ± *SD* of three independent replicates. For all trials, kale was used. Bars sharing a common letter are not significantly different. Total carotenes represent the sum of β-carotene and isomers, total xanthophylls are the sum of lutein, β-cryptoxanthin, violaxanthin and neoxanthin.

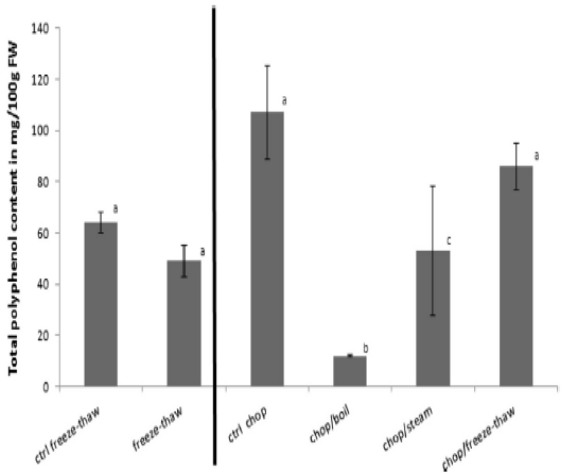

Fig. 2. Effect of different kitchen procedures on total polyphenol content, as determined via the Folin–Ciocalteu assay. The effect of freezing (overnight, −20 °C) combined with thawing (freeze–thaw) was compared to kale crushed under liquid nitrogen and stored until analysis (−80 °C, ctrl freeze–thaw), and the effect of and chopping and boiling (chop/boil), chopping and steaming (chop/steam) as well as chopping and freezing and thawing (chop/freeze–thaw) vs. chopping only (kale cut into ca. 50 cm^2 pieces, than crushed under liquid nitrogen and stored at −80 °C until analysis, ctrl chop) on total phenolics was evaluated. Each bar represents the mean ± *SD* of three independent replicates. For all trials, kale was used. Values sharing a common letter are not significant different.

By comparison, the bioaccessibility of total polyphenols was quite low (possibly due to the cream added to enhance carotenoid bioaccessibility). Major polyphenols found after *in vitro* digestion were neochlorogenic acid and cryptochlorogenic acid. The bioaccessibility of total polyphenols differed within cabbages and plums, and was highest in Kale (6.8%) and lowest in Plum 620 (0.1%, Table 1).

For total carotenoids, total carotenes (mostly beta-carotene) and total xanthophylls (mostly lutein), the cellular uptake did not differ significantly between plums and cabbages. However, total carotenoid uptake was higher for the co-culture cell model than for the Caco-2 cell model ($p < 0.01$, Fig. 3). In both cell models, the cellular uptake of total carotenoids differed significantly according to variety ($p < 0.001$). For cabbages, highest cellular uptake of total

carotenoids was found from Kalorama and lowest from Scots Kale (Fig. 3). In the plum varieties, the highest cellular uptake was found for Ersinger and was lowest for Cherry Plum. The sequence for cellular uptake from the different varieties between the two cell models was similar, but not entirely the same (Fig. 3). Total carotenes were significantly better taken up than total xanthophylls ($p < 0.05$) in both cell models. As the bioaccessibility of polyphenols was already low, no polyphenols could be detected by the UPLC method employed. Indeed, very low absorbance of polyphenols have been reported even from non-compromising systems (Miranda, Deusser, & Evers, 2013).

Following colonic digestion, the recovery of total carotenoids from cabbage varieties was highest in Kalorama (17.2% ± 2.3), and lowest in Scots Kale (3.0% ± 2.0). In plum varieties, the highest recovery was found in Ersinger (24.5% ± 5.2), and lowest in Cherry Plum (6.7% ± 0.8, Fig. 4). Overall, significant differences existed between varieties ($p < 0.01$), but not between plums and cabbages or between xanthophyll and carotene recovery. As the bioaccessibility of polyphenols was already low, no polyphenols could be detected by the UPLC method employed.

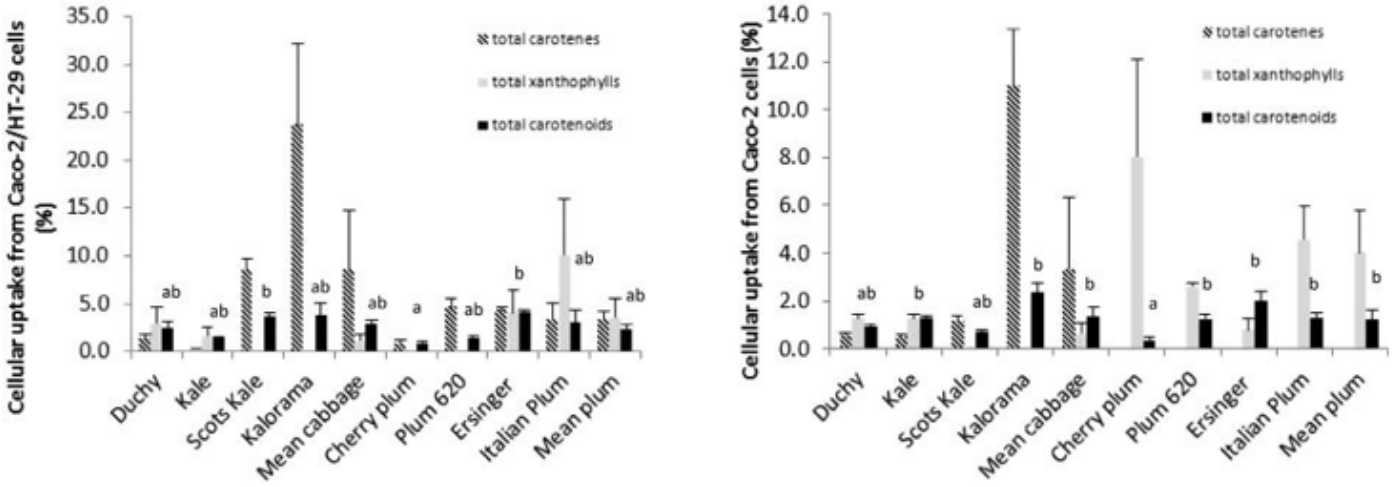

Fig. 3. Fractional cellular uptake of total carotenoids into Caco-2 (right graph) and Caco-2/HT-29-MTX cells (left graph). Xanthophylls (sum of lutein, beta-cryptoxanthin, violaxanthin, neoxanthin) and carotenes (sum of alpha-carotene, beta-carotene) from different cabbage and plum varieties were studied following simulated gastro-intestinal digestion. Each bar presents the mean ± SEM of three independent replicates. Cellular uptake represents the fraction of carotenoid recovered in the cell fraction compared to the original, matrix content. Overall, total carotenoid cellular uptake was higher from the coculture compared to the monoculture ($p < 0.01$), and higher for carotenes compared to xanthophylls ($p < 0.01$), while no significant difference existed between plum and cabbage varieties. Varieties not sharing the same superscript differ significantly in their total carotenoid recovery.

5.4 Discussion

In the present study, we investigated bioavailability aspects of carotenoids and polyphenols from selected plum and cabbage varieties, together with the impact of typical kitchen procedures and colonic fermentation. The findings emphasize the inter-variety variability of bioaccessibility and cellular uptake of phytochemicals, and that cellular uptake (of carotenoids) was not compromised by an additional mucus-layer, which rather increased availability. Better xanthophyll bioaccessibility was counterbalanced by lower cellular uptake, compared to carotenes. A high recovery of carotenoids following colonic recovery was also demonstrated. In addition, carotenoids in cabbage appeared not to be significantly influenced by the selected kitchen procedures, in contrast to total phenolics, which were especially negatively influenced by boiling and steaming.

Kitchen preparations, similar to food processing, may be a double edged sword with respect to phytochemical bioavailability. While it has been highlighted that e.g. maceration and heat application can be beneficial for both carotenoid (Murador, da Cunha, & de Rosso, 2014; Unlu *et al.*, 2007) and polyphenol availability (Bohn, 2014; Bugianesi *et al.*, 2004; Kamilogu *et al.*, 2014), due to improved release from the matrix and solubility during digestion, it also poses the risk for losses through thermal degradation (Fratianni, Cinquanta, & Panfili, 2010; Jaiswal & Abu-Ghanman, 2013). For instance, Fratianni *et al.* (2010) showed that heating orange juice to 85 °C decreased carotenoid content by 50%. In the present study, the decrease of carotenoid content was insignificant, about 4–12%,

possibly due to the protection by the cell walls or other matrix components. Polyphenol content however appears more prone to degradation. For example, fennel total polyphenol content decreased by 85–90% after microwave heating (Jaiswal & Abu-Ghanman, 2013), which is in line with our results (51–89%). Freezing or thawing may likewise result in cell wall maceration, liberating bioactive compounds. In a study done on summer fruits, freezing appeared to increase the detectable total carotenoid content of apricots by 21% and the anthocyanin content of plums by 55% (Leong & Oey, 2012). In the present study, freezing also appeared, though not significantly, to increase the total carotenoid content of Kale, while the total polyphenol content was significantly decreased.

The observation that only polyphenols but not carotenoids were negatively influenced by boiling/steaming, though observed only within a single matrix, may be associated with their additional degradation by enzymes prior to their deactivation, such as polyphenol-oxidase (Schinella *et al.*, 2010; Terefe *et al.*, 2015). The decrease of polyphenol content due to heating processes was already observed in another study (Xu *et al.*, 2014). The degradation was significantly lower (38%) in the case of steaming, emphasizing, as pointed out earlier, that direct avoidance of hot water may be superior to preserve polyphenol content (Faller & Fialho, 2009). Indeed, phenolic compounds are highly hydrophilic molecules and losses through leaching might also occur (Rawson, Hossain, Patras, Tuohy, & Brunton, 2013).

Bioaccessibility is a pre-requisite for later absorption and is a crucial step for bioavailability (Lemmens *et al.*, 2014). The present results indicate that carotenoids from plum varieties may be slightly better micellarized than carotenoids from cabbage varieties, although results did not reach significance in the chosen model with varieties as random factor, and their total amount in plums (Table 1) and therefore also their micellarized amounts were lower as opposed to cabbages. Schweiggert and Carle (in press) showed that carotenoids in carrots and tomatoes were present in chromoplast in large crystalloid form, while in mango and papaya they occurred in globular and tubular substructures in liquid-crystallize form, with the latter form showing higher bioaccessibility. It can only be speculated which form of carotenoids is present in cabbage and plum, but due to their association with chloroplasts and chromoplasts, respectively, their presence as crystalloids and in liquid-crystallized form, respectively, can be hypothesized (Schweiggert & Carle, in press). The presence of carotenoids in crystalloid form such as from cabbage would limit the transfer to the aqueous phase during extraction (Palmero *et al.*, 2014). The fact that in the present study, we did not find large, significant differences between the bioaccessibility may be in part due to the relatively large intra-individual variation of carotenoids within both cabbages and plums. The difference may also in part be explained by the varying carotenoid profile encountered, as xanthophylls are typically better micellarized than carotenes (Furr & Clark, 1997), which was also found in the present study, and higher amounts of xanthophylls were encountered in the plum varieties.

Polyphenol bioaccessibility was quite low, and most likely compromised due to our *in vitro* digestion model, employing cream as a micellarization enhancer, so that only some polyphenols were detected following digestion. Earlier reports have stated that the addition of milk can indeed reduce polyphenol content, due to the formation of complexes with proteins (Serafini *et al.*, 2009). However, some studies have shown that the addition of dietary fat may also increase the bioaccessibility of polyphenols (Ortega, Reguant, Romero, Macia, & Motilva, 2009), especially of the more apolar ones such as aglycons, which likewise may require micellarization. In this respect, other lipid choices such as oils may have resulted in superior bioaccessibility of both carotenoids and polyphenols, given that they can be sufficiently emulsified during digestion. Nonetheless, some studies have shown that polyphenol content decreased by 40–70% after intestinal digestion, and that the remaining polyphenols were mostly found in the non-absorbable fraction (Bouayed, Deusser, Hoffmann, & Bohn, 2012; Correa-Betanzo *et al.*, 2014). This non-absorbable fraction is then, together with the non-absorbed or re-excreted polyphenols, under physiological conditions, undergoing further colonic metabolism, leading to the production of additional metabolites (Mosele, Macia, Romero, Motilva, & Rubio, 2015). This colonic metabolism is an important step in polyphenol bioavailability and normal *in vitro* digestion without colonic fermentation would likely underestimate polyphenol bioaccessibility.

The cellular uptake of total carotenoids was relatively low compared to previous studies (Biehler, Hoffmann, Krause, & Bohn, 2011) and varied between 0.3% and 4.1%. Contrary to

bioaccessibility, carotenes were taken up significantly better than xanthophylls, despite the fact that xanthophylls may reside at the surface of the mixed micelles (while carotenes are located in the core), making them potentially more available for cellular uptake (Sy *et al.*, 2012). However, as transporters may be involved in carotenoid absorption (CD36, SRB-1, NPL-1P), it cannot be excluded that certain carotenes may be taken up preferably (During, Doraiswamy, & Harrison, 2008; Reboul & Borel, 2011), as also observed previously (Biehler *et al.*, 2011).

In the present study, we also tested whether the co-culture cell model including the mucus producing cell line HT-29-MTX may hamper carotenoid uptake, due to the potential additional barrier introduced. However, carotenoid uptake from the coculture rather increased compared to the monoculture cell model. It can thus be assumed that the polar micelles were not hindered by the additional mucus layer. Contradicting results were reported in the literature; for example, iron absorption from highly bioavailable iron digests was negatively influenced by the mucus layer, while iron absorption from low bioavailable iron digests was not influenced by the mucus layer (Mahler, Shuler, & Glahn, 2009). Another study showed that the mucus layer was not influencing the absorption of curcumin (Guri, Gülseren, & Corredig, 2013), a rather apolar polyphenol. Whether the coculture may even foster a better uptake than the monoculture remains speculative – it cannot be excluded that the mucus produced by the HT-29-MTX cells aid to a superior binding of the mixed micelles to the cells, facilitating the uptake of carotenoids, e.g. via increasing the unstirred water-layer area.

With respect to colonic fermentation, blank samples contained relatively high carotenoid concentrations, especially zeaxanthin, resulting from the faeces of pigs, possibly originating from corn containing feedingstuffs. However, carotenoids were quantifiable after colonic fermentation of our digesta and recovery was between 4% and 25% for total carotenoids. Colonic fermentation of the digesta did not considerably affect total carotenoid content or profile, although the overall recovery was low. Similar results were observed in another study with green leafy vegetables, were the colonic recovery of beta-carotene and lutein varied between 2% and 11% (Serrano, Goni, & Saura-Calixto, 2005). Care should be taken not to over-interpret results, as pig microflora certainly shows some differences to human microflora, though the present aim was to find relative differences between the matrices, and earlier studies on bioactive compounds have employed pig faeces as a model for human fermentation (Labib, Hummel, Richling, Humpf, & Schreier, 2006). Nevertheless, data on colonic fermentation of both carotenoids and polyphenols, their resulting structures and their bioactivity are perceived as a gap of knowledge (Bohn *et al.*, 2015). To our knowledge, no reports on carotenoid degradation following colonic fermentation of cabbage and plums exist, and future studies in this domain would be much warranted, as also colonic uptake of carotenoids cannot be excluded.

5.5 Conclusions

When employing kitchen heating procedures, those requiring less liquid such as steaming were superior in preserving bioactive constituents. Following simulated digestion, the bioaccessible fraction varied considerably from the native profile and from variety to variety, with a similar fractional bioaccessibility and cellular uptake for plums and cabbages. While xanthophylls showed a higher bioaccessibility, cellular uptake of carotenes was higher, which may be related to specific uptake transporters. The use of cream as a fat source to increase carotenoid bioaccessibility compromised polyphenol bioaccessibility. Finally, it appeared that an additional layer of mucus as produced in the present study by mucus producing cells (HT-29-MTX) did not compromise carotenoid uptake, and a large proportion of carotenoids remained intact following colonic fermentation, which warrants further studies to investigate potential colonic availability.

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Chapter 6: Inflammation Related Responses of Intestinal Cells to Plum and Cabbage Digesta with Differential Carotenoid and Polyphenol Profiles Following Simulated Gastro-Intestinal Digestion

In this chapter 6 we have investigated the impact of carotenoids and polyphenols on inflammation related responses of intestinal cells. Therefore, we used a Caco-2 cell model and a triple-culture composed of a coculture of Caco-2/HT-29 MTX cells and THP-1 cells. The results concluded in an article entitled: Inflammation Related Responses of Intestinal Cells to Plum and Cabbage Digesta with Differential Carotenoid and Polyphenol Profiles Following Simulated Gastro-Intestinal Digestion, Kaulmann, A., Legay, S., Schneider, Y.J., Hoffmann, L., Bohn, T., Mol.Nutr.Food Res. 2016, 60, 992-1005.

Inflammation Related Responses of Intestinal Cells to Plum and Cabbage Digesta with Differential Carotenoid and Polyphenol Profiles Following Simulated Gastro-Intestinal Digestion

Abstract:

Scope: Plums/cabbages represent fruits/vegetables rich in carotenoids and polyphenols, and have been associated with anti-inflammatory properties.

Methods and results: We tested four plum (Italian Plum, Plum 620, Ersinger, and Cherry Plum) and cabbage varieties (Duchy, Kalorama, Kale, Scots Kale) with contrasting carotenoid/polyphenol content for their capability to alter inflammation/oxidative stress following simulated gastrointestinal digestion. Digesta were exposed to Caco-2(TC-7) and to a triple-culture(Caco-2/HT-29-MTX (90:10 v/v) including THP-1 like macrophages), stimulated to induce inflammation (10 µg/mL LPS, 100 ng/mL TNF-α, 25 ng/mL IL-1-β for 24 h, the last 18 h with digesta). Endpoints investigated included IL-6, IL-8, PGE-2, NO (all ELISA), NF-κB, MAPK, IL-6, IL-8, iNOS, Nrf2, COX-2 (real-time-PCR) and Nrf2 (immunostaining). IL-6 secretion was reduced in THP-1 cells by Scots Kale and Kalorama (up to 22%, $p<0.05$), and IL-8 secretion in the coculture (up to 35% in plums, $p<0.05$). This was accompanied by decreased NF-κB expressions in THP-1 cells (up to 30%, $p<0.05$). Nrf2 translocation to the nucleus was partly reduced by plums and cabbages (up to 40% ($p<0.05$).

Conclusions: Some varieties, especially in the triple-culture, reduced inflammation, though this was unrelated to concentrations of carotenoids/polyphenols. The potential of phytochemical-rich fruits and vegetables to ameliorate gastrointestinal inflammation should be further investigated.

6.1 Introduction

Diets rich in fruits and vegetables are recommended by many health organizations [1], and epidemiological studies have shown that their intake can reduce the incidence of cardiovascular diseases [2], cancer [3] and type II diabetes [4]. Many of these diseases are characterized by a low but chronic and systemic inflammatory component [5, 6]. It has been suggested that the intake of fruits and vegetables may reduce inflammation and oxidative stress via altering signal transduction cascades, which influence gene expression [7–9]. In fact, several plant food constituents, including polyphenols [10], carotenoids [11], anti-oxidant vitamins such as C [12] and E [13], have shown to alter gene expression. Therefore, many countries, including Luxembourg, are promoting fruit and vegetable intake [14]. Plums and cabbages are frequently consumed, with 8 and 7 kg per year and capita, respectively [15]. They also contain relatively high amounts of carotenoids (2 mg/100 g and 13.3 mg/100 g, respectively) and polyphenols (151 mg/100 g and 27 mg/100 g, respectively) [16]. As their concentration typically decreases following ingestion, gastric and small intestinal digestion, cellular uptake, and further biodistribution, it could be proposed that these compounds may be most bioactive in the gastrointestinal tract.

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), have been increasingly diagnosed in the past decades [17], especially in Westernized Countries. CD can affect any part of the digestive tract, while UC mostly affects the colon and the rectum. Both diseases are characterized by abdominal cramps, fever, weight loss and increased inflammation of the intestinal epithelium [18], often

observed together with increased markers of oxidative stress, such as 8-hydroxy-deoxy-guanosine (8-OhdG) [19].

ROS are a normal by-product of aerobic respiration, playing an important role in several physiological processes, such as phagocyte defence. They may in addition act as messenger molecules [20]. However, they also play a role in pathological processes such as inflammation [20, 21]. Therefore, aerobic organisms rely on defence mechanisms, composed of endogenous enzymes (e.g. glutathione reductase, superoxide dismutase) and compounds (e.g. glutathione, uric acid) [22]. However, they also require exogenous antioxidants, e.g. vitamin C or E, or phytochemicals such as carotenoids and polyphenols, though the latter 2 are not essential in a strict nutritional sense. ROS can activate several intracellular signaling cascades including the nuclear transcription factor NF-κB or mitogen-activated protein kinase (MAPK), which then result in the expression of pro-inflammatory cytokines or chemokines [23] such as IL-13, IL-6, IL-8 (an important chemoattractant of neutrophils in IBD), further aggravating oxidative stress and inflammation.

The etiology of UC and CD are still not well understood, but it appears that they result from a combination of environmental factors, genetic predisposition, intestinal microbiota changes and immunological factors [24]. The treatment for the worst cases is surgery, but mostly patients are treated with medical drugs such as steroids, non-steroidal anti-inflammatory drugs or immune selective anti-inflammatory derivatives. However, these strategies were shown to have severe side effects [25,26], such as vomiting, liver pain, loss of appetite, and liver damage

[17]. Therefore, it is prudent to seek alternative strategies that can be employed to prevent and treat IBD, such as a dietary approach, which may be a meaningful adjuvant candidate for IBD therapy.

Carotenoids and polyphenols could constitute such alternative candidates, as some studies have demonstrated that they possess anti-oxidant and anti-inflammatory properties [11,27–29]. It has been shown that carotenoids and polyphenols can decrease the activation of NF-κB, thus downregulating downstream genes such as cyclooxygenase- 2 (COX-2) and iNOS, ultimately decreasing the secretion of pro-inflammatory cytokines and chemokines [11,30].

The aim of this study was to investigate whether the intake of plums and cabbages, as examples of commonly consumed fruits and vegetables containing carotenoids and polyphenols, could affect inflammatory processes in the gut epithelium. For this purpose, a variety of plum and cabbages with contrasting carotenoid and polyphenol profiles as investigated previously [16] was chosen. Gastrointestinal digestion was simulated, followed by employing different cell models, a monoculture with Caco-2 cells, and a triple-culture model consisting of Caco-2, HT-29-MTX and THP-1 cells resembling macrophages (typically aggravating inflammatory responses). Inflammatory markers (IL-8, IL-6, nitric oxide and PGE-2), as well as cellular signaling cascades (NF-κB, MAPK and Nrf2) were studied following inflammatory stimulation.

6.2 Materials and methods

6.2.1 Chemicals

All products used were of analytical grade or superior. Unless otherwise stated, all chemicals, including interleukin-1β (IL-1β), lipopolysaccharides (LPS), arachidonic acid and resazurine, were procured from Sigma-Aldrich (St. Louis, MO, USA). The penicillin/streptomycin mixture and non-essential amino acids were procured from Gibco (Karlsruhe, Germany) and Invitrogen (Camarillo, CA, USA). Tumor necrosis factor-α (TNF-α) was procured from BD Biosciences (Erem-bodegem, Belgium). Milli-Q water (18 MΩ) was prepared with a purification system from Millipore (Brussels, Belgium) and was used throughout the experiments.

6.2.2 Plum and cabbage varieties and gastrointestinal digestion

Cabbages and plums were procured and analysed for major macro-and micro-constituents as described earlier [16]. In brief, 27 cabbage and 17 plum varieties were obtained from Luxembourgish supermarkets or farmers, and their macro-and micro-constituents, including phytochemicals, were determined. Varieties rich in carotenoids, polyphenols, or both were retained for further analyses: *Brassicaceae* varieties (Duchy - *Brassica oleracea var. capitaf. acuta*; Kalorama - *Brassica oleracea convar. capitata var. alba*; Kale - *Brassica oleracea var. sabellica*; Scots Kale - *Brassica oleracea var. sabellica*) and plums (Italian Plum – *Prunus cocomilla*; plum 620 - *Prunus domestica*; Ersinger - *Prunus domestica*; Cherry Plum – *Prunus cerasifera*) were chosen due to their contrasting content of carotenoids and polyphenols (Table 1), and are designated in the following as Duchy +/–, Kalorama –/–, Kale

+/+, Scots Kale –/+; and Italian Plum +/+, Plum 620 –/+, Ersinger –/– and Cherry Plum +/–, in order to indicate varieties rich (+) or poor (–) in carotenoids and polyphenols, respectively.

All *Prunus spp*. and *Brassicaceae* varieties were lyophilized (Christ freeze dryer, Thermo Fisher Scientific, Geel, Belgium) for 24 h, then pulverized with a grinder (Motor Grinder RM 200, Retsch, Aarteselaar, Belgium) and stored in 50 mL plastic centrifuge tubes at –20°C until analysis.

An in-vitro digestion was performed as described earlier [31], employing both the gastric and the small intestinal phases, containing the tested cultivars and a certain amount of cream milk required for assuring optimal carotenoid solubilisation and bioaccessibility (Table 2). Performance of bioaccessibility and cellular uptake were carried out previously, employing an HPLC technique, and results are reported elsewhere [16].

Table 1: Native content of selected phytochemicals and macronutrients of different cabbage and plum varieties tested in this study (taken from Kaulmann et al. [16]).

Plums/ Cabbages	Native content in mg/100 g FW			Native content in g/100 g FW		Antioxidant test		Bioaccessibility	Cellular uptake total carotenoids
	Total carotenoids high: (+), low (-)	Total polyphenols high: (+), low (-)	Vitamin C	Dietary fibre	Total sugar	ABTS mg VCE/100g	FRAP µmol Fe(II)/100g	Total carotenoids (%)	Total carotenoids (%)
CABBAGES									
Duchy	8.1 (+)	3.3 (-)	77.2	2.24	n.i.	166	569	6.6	0.96
Kale	13 (+)	27 (+)	75.9	1.49	n.i.	190	1493	6.8	1.29
Scots Kale	1.0 (-)	6.8 (+)	56.6	2.01	n.i.	381	3212	0.9	0.68
Kalorama	0.1 (-)	0.1 (-)	36.0	3.72	n.i.	28.9	774	3.6	2.38
PLUMS									
Cherry Plum	2.0 (+)	15 (-)	48.8	1.15	8.97	230	1687	3.6	0.32
Plum 620	0.6 (-)	123 (+)	8.69	1.27	12.6	233	1451	11.3	1.23
Ersinger	0.5 (-)	25 (-)	41.5	0.49	12.2	270	913	9.0	2.02
Italian plum	2.0 (+)	83 (+)	24.6	0.95	12.1	328	2264	3.4	1.32

Each value presents the mean of three replicate samples. FW = fresh weight. n.i. = no information
ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
FRAP: Ferric ion reducing antioxidant power
Total bioaccessibility of polyphenols could not be assessed due to lack of standards and many changes during digestion

6.2.3 Cell cultures

The TC-7 subclone (ATCC Number: HTB-37, Manassas, VA, USA) of the Caco-2 parental cell line was derived from a tumor isolated by J. Fogh [32], and was a generous gift from Monique Rousset (Nancy University, France). The HT-29-MTX cell line (ATCC number: HTB-38) was a kind gift from Dr. Técla Lesuffleur (Institut National de la Santéet de la Recherche Médicale, INSERM UMR S 938, Paris, France). The THP-1 cell line was also obtained from ATCC. Cultures were routinely maintained in 75 cm^2 plastic flasks (Nunclon™, Nunc, Denmark) at 37°C and 10% CO2 in a CB-210 CO2 Incubator (Binder GmbH, Tuttlingen, Germany). Caco-2 and HT-29-MTX cells were grown in Dulbecco's modified eagle medium (DMEM + GlutaMAX™,

Gibco), supplemented with 1% non-essential amino acids, 10% heat-inactivated fetal bovine serum (Gibco), 1% of a mixture of penicillin and streptomycin (10 000 units and 10 mg/mL, respectively) and subcultured weekly after reaching ca. 80% confluence. THP-1 cells were grown in RPMI 1640 medium (RPMI + GlutaMAX™, Gibco), supplemented with 10% (v/v) FBS Superior (Millipore). The differentiation of the THP-1 cells into macrophage-like cells was achieved following the protocol of Klein *et al.* [33]. These cell lines were chosen for several reasons. Caco-2 cells have been employed in many previous studies as an established model of the intestinal epithelium, while HT-29 MTX cells produce mucus, which is considered being more realistic [34], *i.e.* closer to the in-vivo condition. THP-1 cells simulate, when differentiated, macrophages, which are expected to infiltrate during IBD into the epithelium, but are easier to grow and handle than primary macrophages *in vitro* [35].

6.2.4 Cell viability

To test the viability of the cells following exposure to inflammatory stimuli and digesta, the resazurine assay was employed [36]. Cells were exposed to pro-inflammatory cytokines in order to induce inflammation, *i.e.* 100 ng/mL TNF-α together with 25 ng/mL IL-1β and 10 µg/mL LPS were spiked into the apical and also, for the triple-culture, into the baso-lateral compartment, for a total of 24 h. During the hours 6–24, cells were also exposed, but in the apical compartment only, to the respective plum and cabbage digesta (1:8, digesta:medium, v/v).

After the treatments, cells were washed with basal medium (DMEM + GlutaMAX™, supplemented with 1% non-essential amino acids, 10% heat-inactivated fetal bovine serum (GIBCO™), 1% of a mixture of

penicillin (10 000 units) and streptomycin (10 mg/mL), respectively, and incubated with medium containing 400 µM resazurine in the dark for 2 h (37°C, 90% air, 10% CO_2). Fluorescence was measured by a BioTek Synergy 2 plate-reader (Colmar, France), with excitation at 530 nm (530 ± 25 nm filter) and emission recorded at 590 nm (590 ± 25 nm filter). Cell viability was expressed as mean (in %) compared to cells without treatment, which was set to 100%.

6.2.5 Exposure experiments

For the monoculture experiments (Caco-2 cells), cells at passage numbers 91–100 were seeded on BD Falcon cell culture inserts (surface area 1 µm pore size, high pore density PET membranes for 6 well plates, BD Biosciences), at a cell density of $5*10^4$ cells/cm^2. Basal media (2 mL each) was placed in the upper and the lower compartment 15 days before the experiments.

For the triple-culture experiments (Caco-2/HT-29-MTX, 90:10 ratio coculture plus THP-1 cells in the basolateral compartment), cells at passage numbers 74 to 77 for Caco-2, 59 to 62 for HT29-MTX and 17 to 20 for THP-1, were used. The coculture part of the triple-culture (Caco-2/HT-29-MTX,) was seeded on BD Falcon cell culture inserts (surface area 1 µm pore size, high pore density PET membranes for 6 well plates, BD Biosciences) at a cell density of $5 * 10^4$ cells/cm^2, and with 2 mL of basal media in the upper and 2 mL of basal media in the lower compartment, 15 days before the experiments. One day prior to the experiments, THP-1 cells were seeded into new 6 well plates (BD Biosciences), at a cell density of $2.4 * 10^4$ cells/cm^2. Cells were differentiated with phorbol 12-myristate 13-acetate (PMA) into

macrophage-like cells overnight in the incubator at 37°C and 5% CO_2 [33]. On the day of the experiment, the media of the THP-1 cells was removed and 2 mL of basal media was added. Then, the inserts with the coculture part of the triple-culture were also added.

Nrf2 immuno-staining following the monoculture experiments (Caco-2 cells) was achieved with cells at passage numbers 74 to 76, which were seeded into 8 well Nunc™ Lab-Tek™ chambered coverglasses (Thermo Fisher Scientific INC, Massachusetts, USA), at a cell density of 5 * 10^4 cells/cm^2, 15 days prior to experiments. For the coculture (Caco-2, HT-29-MTX, 90:10 ratio) experiment, cells at passage numbers 74 to 76 for Caco-2 and 28 to 30 for HT29-MTX were used. The coculture was seeded on 8 well Nunc™ Lab-Tek™ (Thermo Fisher Scientific INC) chambered coverglasses at a cell density of 5 * 10^4 cells/cm^2, 15 days before experiments.

THP-1 cells alone were not studied as they technically could not be investigated by microscope.

Eleven different treatments were performed for both the monoculture and the triple-culture (Table 2) experiments. To each well, 100 ng/mL of TNF-α, 25 ng/mL of IL-1β and 10 µg/mL LPS was added (Fig. 1), either into the apical compartment (monoculture) or both apical and basolateral compartment (triple-culture). Stimuli and concentrations were chosen according to earlier trials [30,36], in order to maximise inflammation stimuli. Each experiment was performed on three different days with each individual treatment executed in triplicate (in duplicate for Nrf2 immuno-staining).

Table 2. Exposure of 15 day-differentiated Caco-2 cells, Caco-2/HT-29-MTX coculture or triple-culture including 1 day differentiated THP-1 cells to various carotenoid treatments ($n = 3$;$N = 3$ per treatment)

Exposure group	Abbreviation	Treatment of cells [$]
Blank (medium)	Blank (B)	Untreated: no digesta, no inflammatory stimuli or micelles
Blank (medium) and stimuli	B+S	No digesta, stimulation with TNF-α, IL-1β and LPS [*] (24h)
Blank, stimuli and empty digesta[**]	B+S+D	Stimulation with TNF-α, IL1-β and LPS (24 h) and empty digesta (hours 6–24)
Blank, stimuli and Duchy digesta[†]	D	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Duchy (hours 6–24)
Blank, stimuli and Scots Kale digesta	SK	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Scots Kale (hours 6–24)
Blank, stimuli and Kale digesta	K	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Kale (hours 6–24)
Blank, stimuli and Kalorama digesta	K	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Kalorama (hours 6–24)
Blank, stimuli and Cherry Plum digesta	CP	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Cherry Plum (hours 6–24)
Blank, stimuli and Plum 620 digesta	P	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Plum 620 (hours 6–24)
Blank, stimuli and Ersinger digesta	E	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Ersinger (hours 6–24)
Blank, stimuli and Italian plum digesta	IP	Stimulation with TNF-α, IL1-β and LPS (24 h) and digesta containing Italian plum (hours 6–24)

[*] 25 ng/mL IL-1β, 100 ng/mL TNF-α and 10 μg/mL LPS simultaneously.
[**] equals positive control
[†] Digesta (500 mg of dried matrix and 3 g cream in 50 mL) was diluted 1:8 with medium (v/v) before addition on the cells.
[$] THP-1 cells in the basolateral compartment were only treated with stimuli, no addition of digesta to this compartment.

6.2.7 Determination of cytokines and nitric oxide secretion

Secretion of IL-6, IL-8, PGE-2 and NO was expressed as a percentage of the positive control (blank + stimulus + empty digesta). Caco-2 cells as well as HT-29-MTX have been reported to be able to secrete cytokines and NO into both apical and basolateral compartments. For the determination of cytokines, COX-2 activity and NO secretion, the supernatants from the monoculture, the coculture (apical compartment) and THP-1 (basolateral compartment) of the triple-culture were tested.

IL-8 concentration in the supernatant was measured by an enzyme immunoassay kit from BD OptEIA (Becton Dickinson, Le Pont-De-Claix, France), according to the manufacturer's recommendation. The measurement of IL-6 concentration in the supernatant was carried out by the IL-6 (human) enzyme immunoassay kit from Cayman Chemical (Ann Arbor; MI, USA), following the manufacturer's instructions. To induce COX-2 activity, cells were additionally incubated for 10 min with arachidonic acid (10 mM in phosphate buffered saline (PBS)). Concentration of PGE-2α in the supernatant was determined using the PGE-2 enzyme immunoassay kit from Cayman Chemical (Ann Arbor; MI, USA), following the manufacturer's instructions. NO is unstable and is rapidly oxidized into nitrate (NO_3^-) and nitrite (NO_2^-). The measurement of NO (as NO_2^-) was carried out by the NO Assay Kit from Merck Millipore (Merck KGaA, Darmstadt, Germany), which is based on the Griess reagent [36].

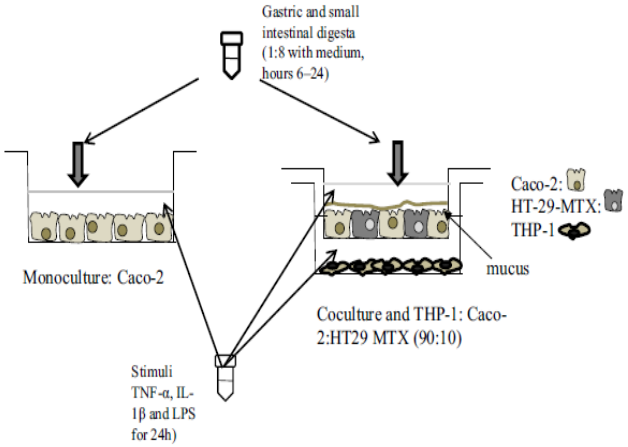


Figure 1. General overview of the experiment protocol.

6.2.8 Real-time PCR

After treatment, cells in the basolateral compartment (THP-1) and in the apical compartment (Caco-2 cells alone and in combination with HT-29-MTX) were investigated separately. Cells were washed twice with PBS, trypsinized with 0.25 % trypsine EDTA (Gibco) and the reaction halted with basal medium. Cells were centrifuged at 200 x g and again washed twice before being resuspended in denaturating guanidine-thiocyanate containing lysis buffer (Qiagen, Leusden, The Netherlands).

Total RNA from samples ($5 * 10^4$ cells/cm^2) was then extracted using the RNeasy Mini Kit with on-column DNase I treatment (Qiagen). Total RNA integrity was assessed using the RNA Nano 6000 assay (Agilent Technologies, Diegem, Belgium) and a 2100 Bioanalyzer (Agilent Technologies). Samples with RINs (RNA integrity number) <7 were excluded from the experiment. RNA purity was assessed measuring the absorbance at 230, 260 and 280 nm using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, Villebon-sur-Yvette, France).

Gene expression results were validated on a subset of seven representative genes (NF-κB, Nrf2, iNOS, IL-6, IL-8, MAPK14, COX-2), using qPCR. Reverse transcription was carried out using the M-MuLV reverse transcriptase (RNase H-), the murine RNase inhibitor (New England Biolabs, Ipswich, MA, USA) and random hexamers (Invitrogen, Carlsbad, NM, USA), following the manufacturers' guidelines. qPCR primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/), with the following criteria: primer size between 18 and 25 base pairs, GC content between 30 and 70%, amplicon size 80 to 150 base pairs, primer annealing temperatures 57– 61°C. Matching primer sets were checked using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html) for unexpected secondary structures. In order to test the specificity of the primers, a BLAST search against the Human genome sequence was carried out. qPCR runs were performed, using technical triplicates and 3 biological replicates on a Viia7 384-well real-time PCR instrument (Life Technologies, Gent, Belgium), with Takyon SYBR Green low ROX (Eurogentec, Seraing, Belgium), following the manufacturer's guidelines. Experiments were carried out following the MIQE guidelines [37], and a melting curve was performed at the end of all runs to assess the specificity of the primers. Primer information and melting characteristics are available in Table 3. The relative expression of a gene of interest was calculated using the Biogazelle (Zulte, Belgium) Qbase+ V2.5 software [38], taking into account multiple reference gene normalization and specific PCR efficiencies. For the monoculture experiment, the GeNorm+ analysis described the B2M and YWHAZ gene couple was chosen as the most suitable for optimal normalization

of the data [39]. For the triple-culture experiment, the GeNorm+ analysis described the 3-actin and YWHAZ gene couple as the most suitable for optimal normalization of the data for the coculture part (Caco-2/HT-29-MTX), and the B2M and YWHAZ gene couple for the THP-1 cells

Table 3. Primers employed in the analysis of mRNA in the present study

Gene symbol	Name	Accession number	Amplicon size (bp)	Amplicon T (°C)	PCR efficiency	Forward primer sequence	Reverse primer sequence	Reference
INOS	Inducible nitric oxide synthase 2	NM_000625	132	83.5	1.931	AAAGACCAGGCTGTCGTTGA	ACGGGACCGGTATTCATTCT	Broadbelt et al. 2007 [63]
IL6	Interleukin 6	NM_000600	90	79	1.961	CTGGATTCAATGAGGACACTT GC	TCAAATCTGTTCTGGAAGGTACTC TAGG	Kalita et al. 2013 [64]
NRF2	Nuclear factor, erythroid 2-like 2	NM_001145413	161	84	1.912	TTCAGCCAGCCCAGCACATC	CGTAGCCGAAGAAACCTCATTGTC	Garbin et al. 2009 [65]
COX2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963	146	84	1.986	TCCACAACCCTCTGCAC	TGCATTCTTTGCCCAGCACT	Novitskiy et al. 2009 [66]
IL8	Chemokine (C-X-C motif) ligand 8 (CXCL8)	NM_000584	92	79.5	1.969	AAGACATACTCCAAACCTTTCC ACC	CAATAATTTCTGTGTTGGCGCA	Kalita et al. 2013 [64]
MAPK14	Mitogen-activated protein kinase	NM_001315	73	85	1.968	CTGCAGCTGCTGAAGTTCTGA	ATTACTCCGGGCCCACAA	Designed
NFKb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_001165412	89	82	1.992	CTGGAAGCACGAATGACAGA	CCTTCTGCTTGCAAATAGGC	Designed
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	NM_000194	94	81.5	1.964	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	Vandesompele et al. 2002 [39]
B-ACTIN	Beta actin	NM_001101	140	87.5	1.96	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA	Vandesompele et al. 2002 [39]
SDHA	Succinate dehydrogenase complex, subunit A	NM_004168	86	79	1.95	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	Vandesompele et al. 2002 [39]
B2M	Beta-2-microglobulin	NM_004048	86	82	1.995	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT	Vandesompele et al. 2002 [39]
YWHAZ	Tyrosine 3-monooxygenase	NM_003406	94	80	2	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	Vandesompele et al. 2002 [39]

6.2.9 Nrf2 labeling, cell fixation and confocal microscope measurements

Cells were washed twice with PBS and were then fixed for 10 min with 100% methanol at –20°C in a freezer. After fixation, cells were washed twice with PBS and in the following incubated for 30 min with 10 % bovine serum albumin (BSA) in PBS (w/v) to block unspecific binding. After blocking, cells were incubated with primary and secondary antibodies for 1 h each at room temperature in the dark. Antibodies were diluted as followed: rabbit anti-human Nrf2 1:200 (ab31163, Abcam, Cambridge, UK), goat anti-rabbit dylight488 1:2000 (AS09 633, Agrisera, Vännas, Sweden). Nuclei were counterstained with Hoechst 33342 (AnaSpec Inc., Fremont, USA) and cellular membranes were stained with RCA-Rhodamine (Vector Laboratories Inc., Burlingame, USA).

A Zeiss LSM 510 Meta with an inverted Zeiss microscope (Axiovert 200 M, Lasers : HeNe 633 nm, HeNe 543 nm, Ar 488 nm and Diode 405 nm; Zeiss, Jena, Germany) was used. Image processing and visualization was by the Zeiss Software ZEN 2011 and Image J (http://rsbweb.nih.gov/ij/). For further statistical analyses, the intensity of the staining of Nrf2 was normalized to the intensity of the nuclei, and the sum of pixel count x intensity was compared for the nuclei only and the entire cells.

6.2.10 Data interpretation

Unless otherwise stated, all data represents mean ± standard error of mean (SEM). All data was evaluated by SPSS vs 19.0 (IBM, Chicago, IL). Normal distribution of results and equality of variance were verified by Q-Q plots and box plots, respectively. For the mRNA

expression, results were square root transformed to achieve a distribution closer to normal. For predicting changes in inflammatory marker secretion (IL-6, IL-8, PGE-2 and NO) and changes in mRNA expression after different treatments of the three cell models, univariate (ANOVA) models were developed with inflammatory marker secretion or mRNA expression as the dependent (observed) variable, and treatment (*i.e*. individual plum and cabbage digesta employed) and day of analysis as the fixed factor. Following significant Fisher-F-tests, all comparisons against the positive control (blank+ stimuli + empty digesta) were carried out by Dunnett's post-hoc test. P-values below 0.05 (2-sided) were considered statistically significant.

6.3 Results

6.3.1 Cell viability, IL-8, IL-6, PGE-2 and NO secretion following digesta exposure

The different treatments did not result in a significant reduction of cell viability of the two different cell models (data not shown). The addition of the inflammatory stimuli (TNF-a, IL-1β and LPS, termed blank + stimuli) to the media of the cultures resulted in a ca. 50, 6 and 70 fold induction of the IL-8 secretion, compared to the unstimulated cells (blank), for the monoculture, coculture and THP-1 cells, respectively. The empty digesta (blank + stimuli + digesta), *i.e*. digesta without cabbages or plums (positive control), resulted in a further ca. 2 fold induction of the IL-8 secretion, compared to the stimuli without digesta (blank + stimuli), at least for the mono-and coculture. While the different cabbage treatments did not show any significant effect on the IL-8 secretion, plums significantly reduced IL-8 secretion in the

monoculture and coculture model (Fig. 2), with no effect on the THP-1 cells.

Contrarily, the inflammatory stimuli did not result in a detectable induction of the IL-6 secretion in the mono-and coculture (Supporting Information Fig. 1), with concentrations staying quite low (Supporting Information Fig. 1), while a ca. 50 fold induction was observed in THP-1 cells (Fig. 3). Certain treatments (Scots Kale, Kalorama) resulted in a significant reduction of the IL-6 secretion compared to the positive controls. Concerning NO, no enhanced secretion was observed following the stimuli. However, following exposure to plum and cabbage digesta, higher NO levels were detected (Fig. 2), though not in the THP-1 cells, perhaps indicating that NO levels did originate from the plant matrix itself.

The PGE-2 level was already high in unstimulated cells, and there was no significant induction or decrease by the inflammatory stimuli or the different treatments (Supporting Information Fig. 3).

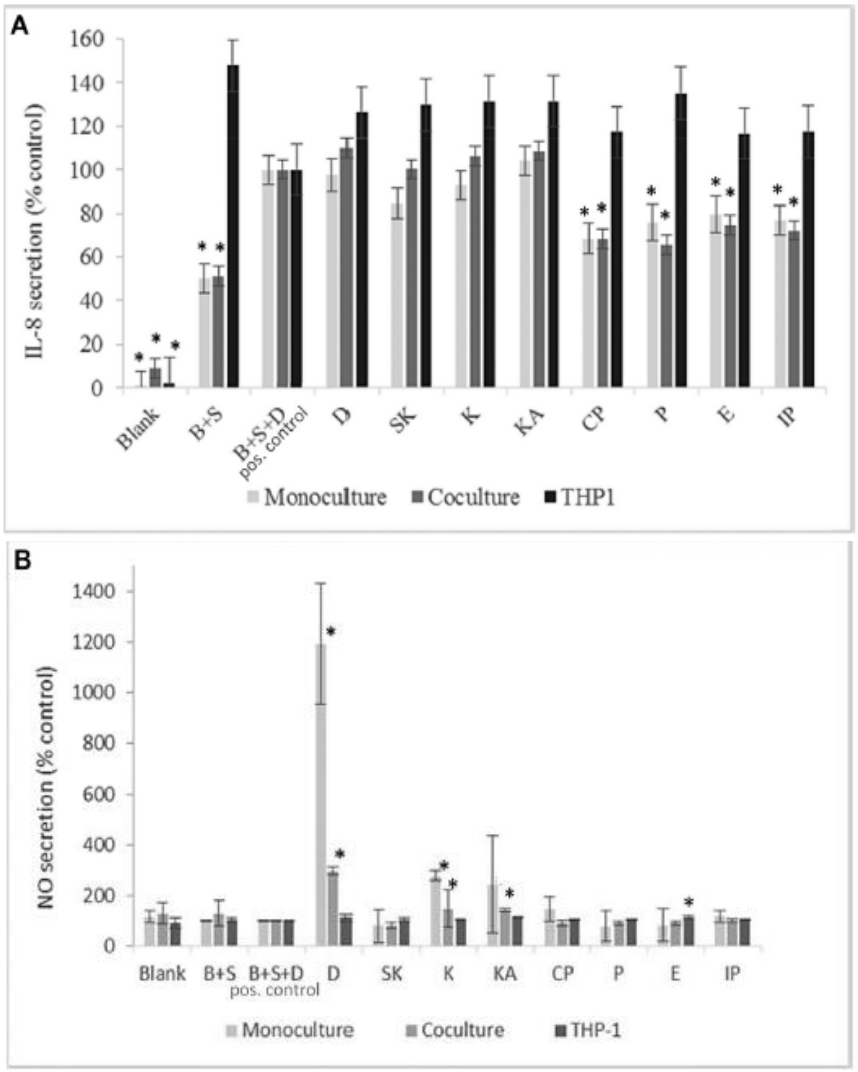


Figure 2. IL- 8 (A) and NO (B) secretion of intestinal cells from three types of cells (Caco-2 cells only, Caco-2/HT-29-MTX plus THP-1 cells), following stimulation with pro-inflammatory agents and exposure to digested cabbage and plum cultivars. Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 μg/mL) for 6 h (both apical and basolateral compartments (THP-1)), before the cells were exposed to various plum/cabbage digesta (only apical compartment) and inflammatory stimuli (both compartments) for another 18 h. IL-8/NO secretion was measured after 24 h in the apical (Caco-2 and HT-29-MTX cells) and the basolateral supernatant (THP-1 cells). Values represent means ± SD of three individual replicates from three different days. *Mean values were significantly different from positive controls (B+S+D = blank + stimuli + empty digesta, which were set as 100%) by Dunnett's test ($p < 0.05$). Cabbages: Duchy-D (+/–), Scots Kale-SK (–/+), Kale-K (+/+), Kalorama-KA (–/–). Plums: Cherry Plum-CP (+/–), Plum-P 620 (–/+), Ersinger-E (–/–), Italian plum-IP (+/+). (+/–) indicates levels of carotenoids and polyphenols, respectively.

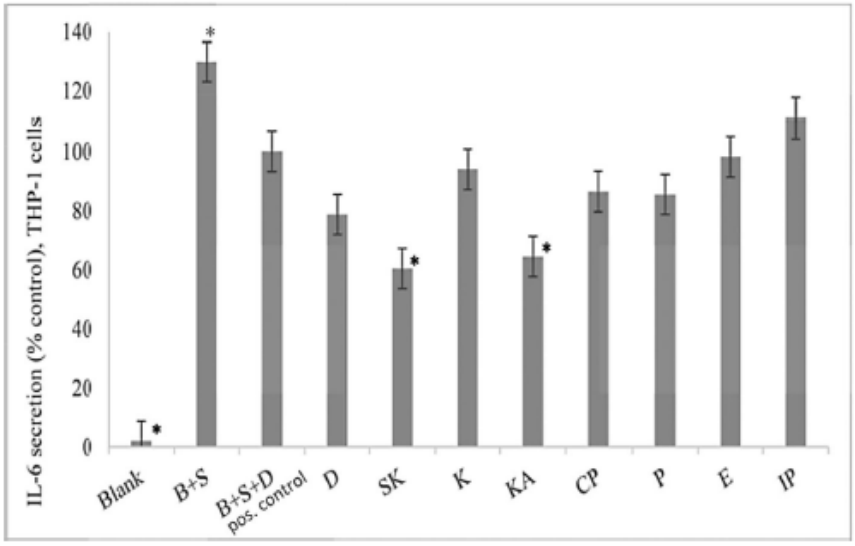


Figure 3. IL– 6 secretion of THP-1 cells as part of the triple-culture (Caco-2/HT-29-MTX plus THP-1 cells), following stimulation with pro-inflammatory agents and exposure to digested cabbage and plum cultivars. Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 μg/mL) for 6 h (both apical and basolateral compartments (THP-1)), before cells were exposed to various plum/cabbage digesta (only apical compartment) and inflammatory stimuli (both compartments) for another 18 h. IL-6 secretion was measured after 24 h in the apical or the basolateral supernatant (THP-1). Values represent means ± SD of three individual replicates from three different days. *Mean values were significantly different from positive controls (B+S+D = blank + stimuli + empty digests, which were set as 100%) by Dunnett's test ($p<0.05$). Cabbages: Duchy-D (+/–), Scots Kale-SK (–/+), Kale-K (+/+), Kalorama-KA (–/–). Plums: Cherry Plum-CP (+/–), Plum-P 620 (–/+), Ersinger-E (–/–), Italian plum-IP (+/+). (+/–) indicates level of carotenoids and polyphenols, respectively. No significant changes in the coculture part of the triple-culture were observed (as in the monoculture).

6.3.2 Real-time PCR

The addition of the inflammatory stimuli to the mono-and co-culture cell models did not result in a measurable significant change of the expression level of most of the different genes investigated (COX-2, iNOS, MAPK14, Nrf2, Fig. 4 and Supporting Information Fig. 2). In addition, expression of IL-6 was too low to be interpreted in a statistical approach. Likewise, the different digesta also did not result in a significant reduction compared to the positive control (blank + stimuli + digesta, supplementary Fig. 1), except for Nrf2 (Fig. 4), with digesta containing Ersinger (–/–), Plum 602 (–/+) and Italian Plum (+/+) resulting in a significant decrease of the mRNA level ($p< 0.05$) (Fig. 4). Duchy and Italian Plum significantly reduced the expression of MAPK14

(monoculture), compared to the positive control, though also the positive control already showed a reduced expression, compared to the blank.

Adding the inflammatory stimuli to the THP-1 cells of the triple-culture significantly increased the mRNA expression levels of IL-8 and NF-κB, compared to the blank (unstimulated cells, $p<0.01$). The digesta containing Scots Kale (–/+) and Kalorama (–/–) significantly decreased the mRNA expression level of NF-κB ($p<0.01$) (Fig. 4), compared to the positive controls (blank + stimuli + digesta).

6.3.3 Nrf2 staining

Interestingly, the addition of the inflammatory stimuli did not result in a significantly higher Nrf2 nucleus accumulation in both cell cultures, nor did it significantly alter the total amount of Nrf2 detected (data not shown). Similarly, cabbage digesta resulted in a slight but non-significant decrease of the Nrf2 nucleus accumulation in the monoculture (except for Scots Kale (–/+)), compared to the positive control (blank +stimulus + digesta). For the coculture, the 2 cabbage cultivars Duchy (+/–) and Kale (+/+), and the 2 plum varieties Plum 620 (–/+) and Ersinger (–/– significantly decreased Nrf2 accumulation in the nucleus ($p<0.05$, Fig. 5).

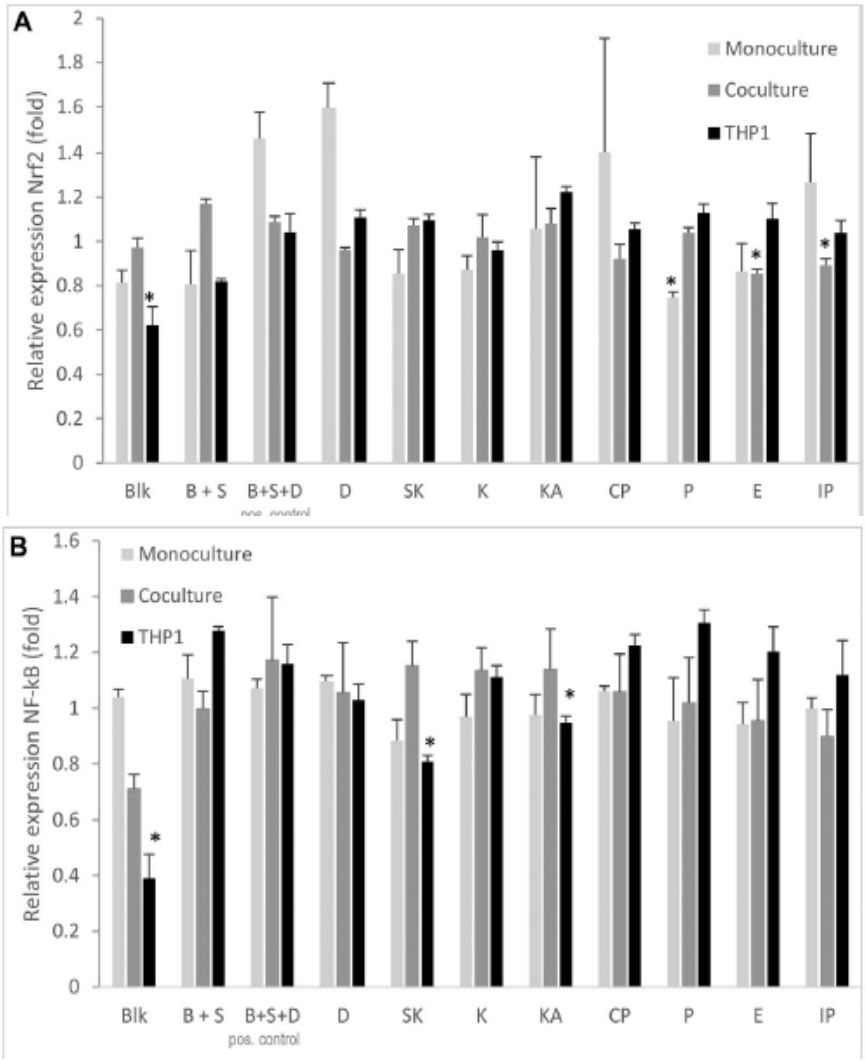


Figure 4. mRNA Expression of Nrf2 (A) and NF-κB (B) of intestinal cells from three types of cells (Caco-2 cells only,Caco-2/HT-29-MTX plus THP1-cells), following stimulation with pro-inflammatory agents and exposure to digested cabbage and plum cultivars. Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 µg/mL) for 6 h (both apical and basolateral compartments (THP-1)), before cells were exposed to various plum/cabbage digesta (only apical compartment) and inflammatory stimuli (both compartments) for another 18 h. mRNA expression expression was measured after 24 h in the apical or the basolateral supernatant (THP-1). Values represent means ± SEM of three different days. * Mean values were significantly different from positive controls (B+S+D= blank + stimuli+ empty digests, which were set as 100 %) by Dunnett's test ($p<0.05$). Cabbages: Duchy-D (+/-), Scots Kale-SK (-/+), Kale-K (+/+), Kalorama-KA (-/-). Plums: Cherry plum-CP (+/-), Plum 620-P (-/+), Ersinger-E (-/-), Italian plum-IP (+/+). (+/-) indicates level of carotenoids and polyphenols, respectively.

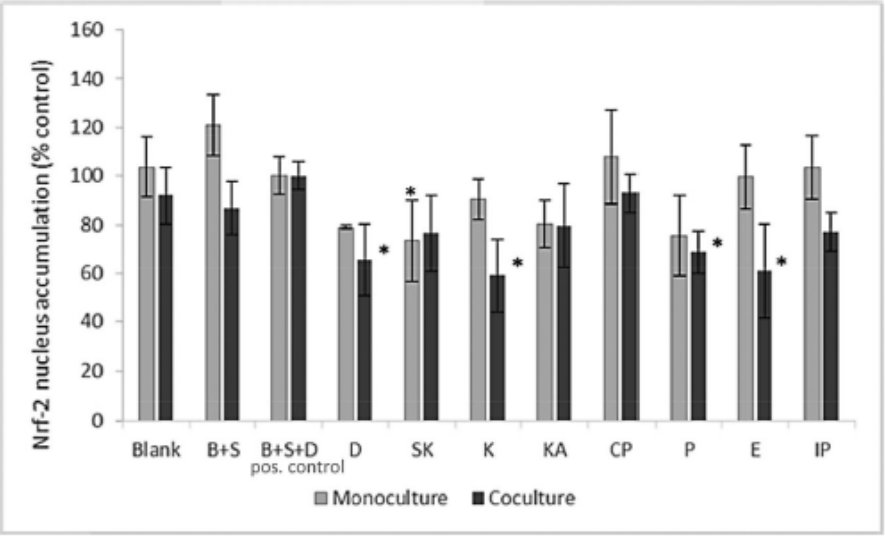


Figure 5. Nrf2 accumulation in the nucleus of intestinal cells (Caco-2 or Caco-2/HT-29-MTX), following stimulation with pro-inflammatory agents and exposure to digested cabbage and plum cultivars. Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 μg/mL) for 6 h, before they were exposed to various treatments and inflammatory stimuli for another 18 h, and the Nrf2 nucleus accumulation in percentage of total Nrf2 was measured after 24 h by means of staining with anti-Nrf2 antibodies. Values represent means ± SD from three individual replicates. *Mean values were significant different from positive controls (B+S+D = blank + stimuli + empty digests, which were set as 100%) by Dunnett's test ($p<0.05$). Cabbages: Duchy-D (+/–), Scots Kale-SK (–/+), Kale-K (+/+), Kalorama-KA (–/–). Plums: Cherry Plum-CP (+/–), Plum-P 620 (–/+), Ersinger-E (–/–), Italian plum-IP (+/+). (+/–) indicates level of carotenoids and polyphenols, respectively.

6.3.4 Bioaccessibility and cellular uptake of carotenoids and polyphenols

Results are presented in Table 1 and were originated from an earlier trial [16]. As can be seen, bioaccessibility of carotenoids ranged between 1.4 and 9.4%, and cellular uptake was still lower. With respect to polyphenol detection, most native polyphenols could no longer be detected by HPLC, and supposedly either reacted to forms for which standards were unavailable, or were precipitated during the digestion, thus total bioaccessibility could not be accurately be determined.

6.4 Discussion

In the present study, we investigated the effects of selected digested plum and cabbage varieties with a contrasting carotenoid and polyphenol content, on different inflammatory mediators (IL-8, IL-6, NO and PGE-2) and related genes (IL-6, IL-8, iNOS and COX-2), as well as on selected intracellular signaling pathways (NF-κB, Nrf2 and MAPK), employing models of the intestinal epithelium. These markers were chosen as they constitute important potential targets involved in the aggravation of IBD [40]. A novel aspect of the present study was the inclusion of macrophage-like cells in a triple-cell culture, in order to constitute a more realistic model of IBD, compared to single cell models.

In general, results demonstrated that the plum and cabbage digesta had somewhat limited effects toward reducing markers of inflammation. Most prominent reductions of inflammatory markers were encountered for IL-8 and IL-6, which were reduced for some cellular models for certain plum/cabbage varieties, and that these changes were in part reflected by a decrease in NF-κB expression. Overall, the coculture (of the triple-culture) tended to be more responsive to plum/cabbage digesta compared to the monoculture. However, reduction of simulated inflammation typically requires that inflammation markers are stimulated to a certain extent. The inflammatory stimuli chosen significantly enhanced several markers related to inflammation, including NF-κB expression and IL-6 secretion (THP-1 cells), as well as IL-8 secretion (all cell models), but did not alter Nrf2, NO, and other markers measured. This stimulation was similar for the mono and the coculture. It is also noteworthy that the empty digesta (without

cabbage/plums) further increased inflammatory markers, such as IL-8 secretion, perhaps due to the negative effects of bile salts as shown earlier [41].

Various studies have shown that the transcription factor NF-κB plays a pivotal role in the upregulation of down-stream genes responsible for secreting pro-inflammatory cytokines and chemokines, e.g. IL-6, IL-8, and interferons (IFN) [42–44]. Reducing these markers is expected to diminish inflammation and further tissue damage. Anti-oxidant phytochemicals, such as carotenoids and polyphenols, have been recently promoted as potential beneficial compounds against IBD [11,45], as both exert anti-oxidant and anti-inflammatory properties, via altering intracellular signaling cascades. For example, IL-8 was reduced by 45% in human serum, following berry anthocyanin intake [46]. Romier *et al.* [30] showed that a polyphenol-rich pomegranate extract decreased ERK1/2 and NF-κB activation, as well as the secretion of IL-8, NO and PGE-2, in a Caco-2 model of inflammation. In another trial, sera of rats fed with blueberries resulted in decreased production of IL-6 mRNA in macrophages, reducing NF-κB and MAPK expression [47]. Similar effects were also noted for the more apolar carotenoids. Lycopene addition to LPS-stimulated macrophages reduced IL-6 and NO secretion, as well as mRNA expression and NF-κB translocation and ERK1/2 phosphorylation [48], and a lycopene-rich tomato extract inhibited LPS-induced NF-κB activation in IEC-18 small intestinal cells [49]. In the present study, a reduction of IL-6 protein levels and possibly associated NF-κB mRNA expression by some cabbage varieties (Scots Kale (–/+) and Kalorama (–/–)) was observed, though only in the THP-1 part of the triple-culture. Concerning IL-8, plum, but not cabbage digesta, reduced

IL-8 protein levels by 23–37% in the mono and coculture part of the triple-culture. All plum varieties contained a higher ratio of polyphenols to carotenoids than the cabbages, though not higher absolute concentrations, making it unlikely to associate their concentrations with observed effects. Perhaps matrix effects could have played a role, such as higher gelling properties and water solubility of the pectin-rich plum matrices [50], resulting in better bioaccessibility of bioactive constituents, though this was not observed for carotenoids or polyphenols in the present study (Table 2). Interestingly, no reduction in IL-8 mRNA expression was detected following digesta exposure. It is possible that the increase upon stimulation has only been transient in time, or that it took a longer time for a full response from the addition of digesta in the apical compartment to upregulation in the THP-1 cells (basolateral compartment). Similar considerations may have applied to iNOS expression and secretion, another downstream target of NF-κB, which also was absent in THP-1 cells.

MAPK activation also plays a role in IBD, and can lead to pro-inflammatory cytokine production, such as IL-6, IL-8, and COX-2, and activation of NF-κB [51]. Several polyphenols, e.g. citrus fruit flavonoids, have shown to reduce MAPK signaling, ultimately reducing iNOS and COX-2 activity in macrophages [52]. In the present study, the digesta did only partially reduce MAPK expression, not significantly affecting iNOS or COX-2 mRNA expression in any of the cell models, possibly as stimulation of inflammation was low in the first place (Supporting Information Fig. 2). Contrarily, NO secretion appeared increased in the mono and coculture cells by several cabbage varieties. The relatively high nitrate levels of cabbage (51 mg/100 g) [53] may

have resulted in positive results with the Griess test, which is corroborated by unaltered iNOS expression levels.

Nrf2 is likewise an important transcription factor, involved in cellular stress response, maintaining physiological concentrations of ROS and reactive nitrogen species, including e.g. hydrogen peroxide and NO [54]. In a previous trial, Nrf2 was impaired in subjects suffering from UC and CD [55]. As with NF-κB, polyphenols and carotenoids have shown to act on Nrf2. For instance, a study on HepG2 (human liver carcinoma) cells, lycopene increased Nrf2 activation, *i.e*. translocation from cytosol to nucleus [56]. In another study, epigallocatechin-3-gallate also increased activation of Nrf2 in lung tissue of rats [57]. Contrariwise (at least for the coculture), both cabbages (Duchy (+/–), Kale (+/+)), and plums (Plum 620 (-/+) and Ersinger (–/–)), decreased Nrf2 translocation from the cytosol to the nucleus (Fig. 5). Ersinger (–/–) and Italian Plum (+/+) also decreased Nrf2 mRNA expression in the coculture. It is possible that carotenoids and polyphenols exhibit pro-oxidant properties at higher concentrations [58], though within food matrixes, this pro-oxidant phenomenon appears reduced [22], and amounts used in this study were surely physiological. Another possibility is that carotenoids and polyphenols reduced the general ROS state of the cells, diminishing the need to produce anti-oxidant active molecules, reducing Nrf2 translocation.

The THP-1 cells of the triple-cell culture showed a more pronounced response to certain inflammatory stimuli (NF-κB expression, IL-6 secretion) than the mono- and coculture, possibly as immune cells respond stronger to inflammation than enterocyte type cells. On the other hand, if a certain crosstalk occurred between the apical and the

basolateral part of the triple-culture, the effects appeared rather limited, as the mono- and the coculture of (the triple-culture) behaved rather similar, except for Nrf2. Again, it is possible that the time period was too short to allow for sufficient diffusion of digesta constituents to the THP-1 cells in the basolateral compartment. However, different absorption capabilities between the Caco-2 cells and the coculture may also have played a role, as a somewhat higher cellular uptake of e.g. carotenoids for the coculture was observed previously [31].

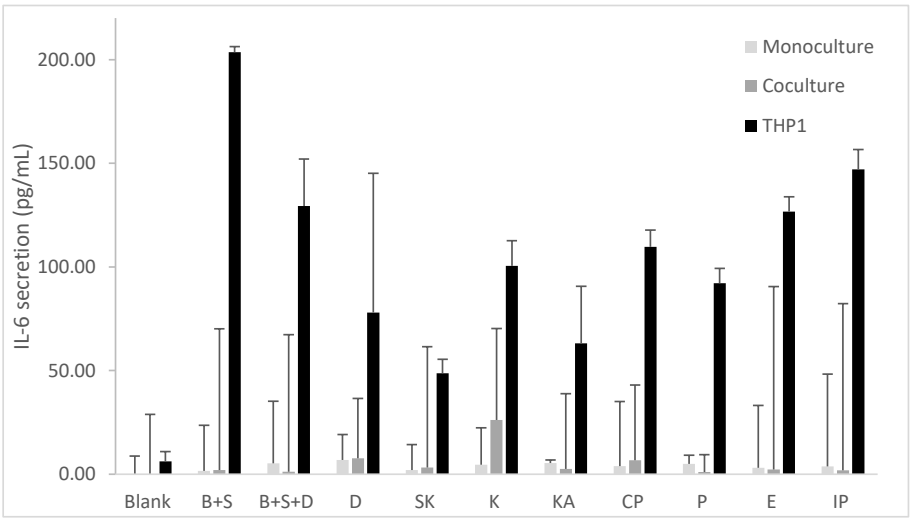
In the present study, the digesta with the greatest impact on inflammatory mediators or signaling cascades (at least in 2 tests), *i.e.* Scots Kale (–/+), Kalorama (–/–), Plum (–/+), and Ersinger (–/–), except for Italian Plum (+/+), did not consistently contain high contents of carotenoids and polyphenols. The potential employment of polyphenols in IBD has been recently emphasized by Martin and Bolling [59], arguing that possibly supplemental (rather than dietary) doses are required to be effective, perhaps related to their limited bioavailability [31]. Thus, it appears that the effects detected in the present study are unlikely to be ascribed to carotenoids or polyphenols alone, but may be related to other compounds. For example, the relatively high vitamin C content of some varieties (57 mg/ 100 g for Scots Kale) could have contributed to the observed effects, also exerting anti-inflammatory properties, as shown by reduced IL-6 secretion in LPS induced monocytes [60], and reduced NF-κB activation in human neuroblastoma cells [61]. In cabbage, glucosinolates could also have played a role in reducing inflammatory markers, as isothiocyanates from cruciferous vegetables may also act toward reducing cytokines in humans [62].

In summary, both cabbage and plum varieties reduced certain markers of inflammation (IL-6, IL-8 and NF-κB) and of oxidative stress (Nrf2), though effects were moderate and could neither be aligned to either cabbages or plums, nor to carotenoids or polyphenols. More studies in this domain, employing fruits and vegetables rich in bioavailable secondary plant compounds, and also further sophisticated models of IBD, are warranted.

The authors declare no conflict of interest.

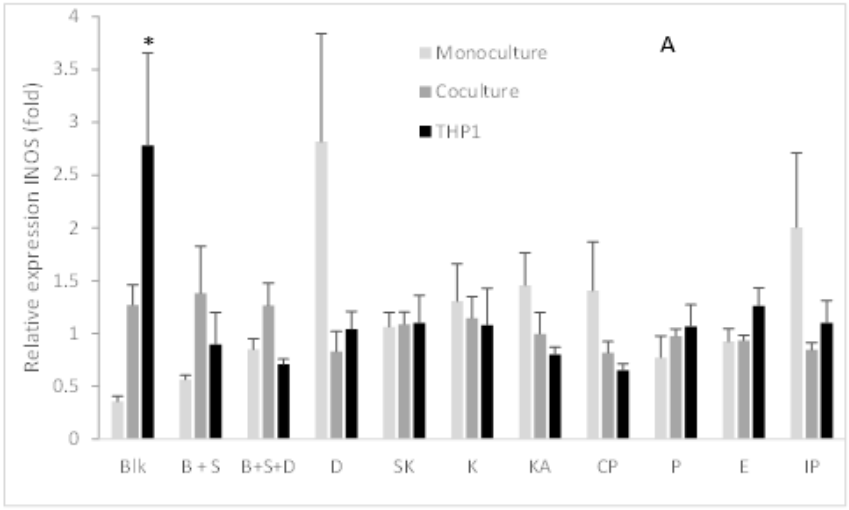
6.5 Supplemental Data

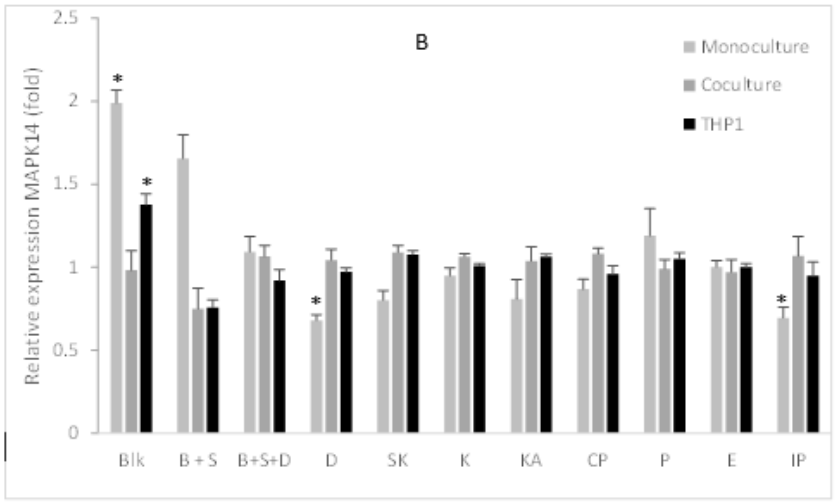
Supplementary figure 1:

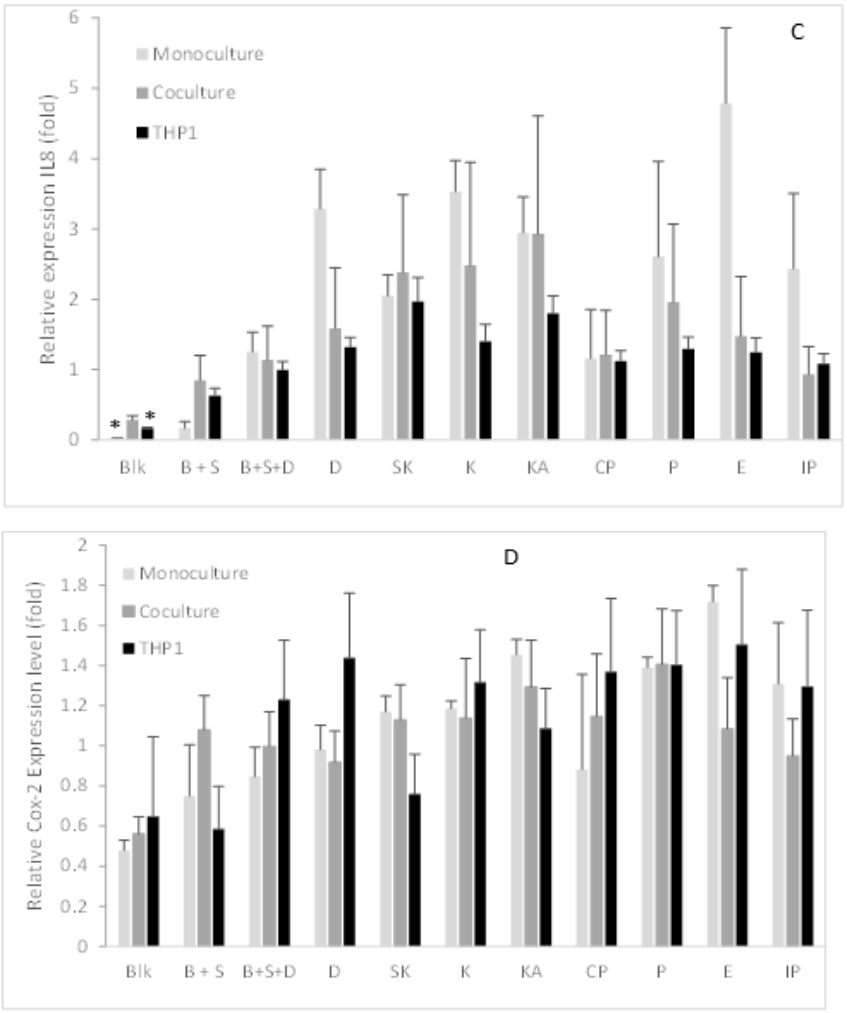


IL– 6 secretion by THP-1 cells as part of the triple culture (Caco-2/HT-29-MTX plus THP-1 cells), as well as by Caco-2 cells (monoculture) and the Caco-2/HT-29-MTX coculture model, following stimulation with pro-inflammatory agents and exposure to digested cabbage and plum cultivars.
Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 µg/mL) for 6 h (both apical and basolateral compartments (THP-1)), before cells were exposed to the various plum/cabbage digesta (only apical compartment) and inflammatory stimuli (both compartments) for another 18 h. IL-6 secretion was measured after 24 h in the apical or the basolateral supernatant (THP-1). Values represent means ± SEM of three individual replicates from three different days. Values represent uncorrected concentrations of IL-6 in the respective supernatant media.
Cabbages: Duchy-D (+/-), Scots Kale-SK (-/+), Kale-K (+/+), Kalorama-KA (-/-). Plums: Cherry Plum-CP (+/-), Plum-P 620 (-/+), Ersinger-E (-/-), Italian plum-IP (+/+). (+/-) indicates level of carotenoids and polyphenols, respectively. B+S and B+S+D (positive control) represents blank plus stimuli without and with digesta, respectively.
No significant differences in the monoculture and coculture models were detected.

Supplementary figure 2.

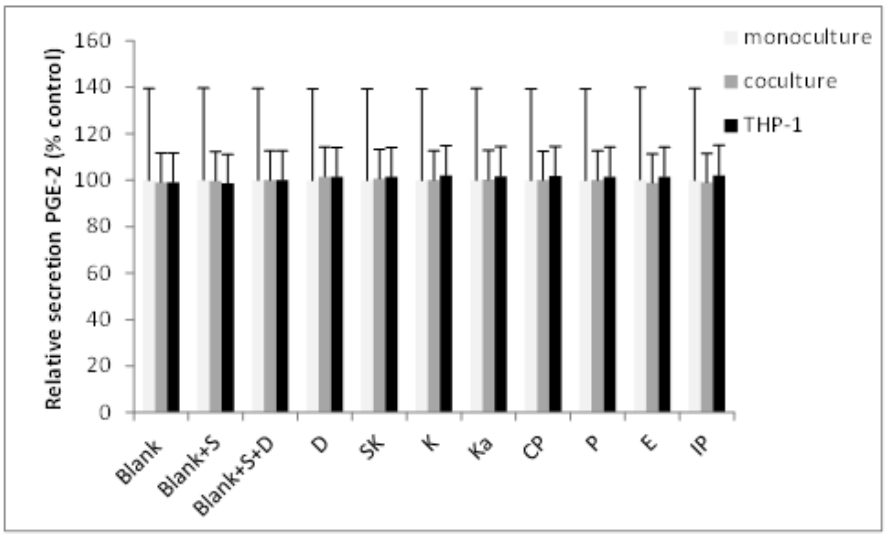






mRNA Expression of, INOS (A), MAPK14 (B), IL-8 (C), and Cox-2 (D), by THP-1 cells as part of the triple culture (Caco-2/HT-29-MTX plus THP-1 cells), as well as by Caco-2 cells (monoculture) and the Caco-2/HT-29-MTX coculture model, following stimulation with pro-inflammatory agents followed by exposure to digested cabbage and plum cultivars.
Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 µg/mL) for 6 h (both apical and basolateral compartments (THP-1)), before cells were exposed to various plum/cabbage digesta (only apical compartment) and inflammatory stimuli (both compartments) for another 18 h. mRNA expression was measured after 24 h in the apical or the basolateral supernatant (THP-1). Values represent means ± SEM of three individual replicates from three different days. Cabbages: Duchy-D (+/-), Scots Kale-SK (-/+), Kale-K (+/+), Kalorama-KA (-/-). Plums: Cherry Plum-CP (+/-), Plum-P 620 (-/+), Ersinger-E (-/-), Italian plum-IP (+/+). (+/-) indicates level of carotenoids and polyphenols, respectively. B+S and B+S+D represents blank plus stimuli without and with digesta, respectively. *indicates stat. sign. differences ($P<0.05$) compared to the positive controls (B+S+D).

Supplementary figure 3.



PGE-2 secretion by THP-1 cells as part of the triple culture (Caco-2/HT-29-MTX plus THP-1 cells), as well as by Caco-2 cells (monoculture) and the Caco-2/HT-29-MTX coculture model, following stimulation with pro-inflammatory agents and exposure to digested cabbage and plum cultivars.

Cells were stimulated for inflammation with a mixture of TNF-α (100 ng/mL), IL-1β (25 ng/mL) and LPS (10 µg/mL) for 6 h (both apical and basolateral compartments (THP-1)), before cells were exposed to various plum/cabbage digesta (only apical compartment) and inflammatory stimuli (both compartments) for another 18 h. To induce COX-2 activity, cells were additionally incubated for 10 min with arachidonic acid (10 mM in phosphate buffered saline (PBS)). PGE-2 secretion was measured after 24 h in the apical or the basolateral supernatant (THP-1). Values represent means ± SEM of three individual replicates from three different days. Cabbages: Duchy-D (+/-), Scots Kale-SK (-/+), Kale-K (+/+), Kalorama-KA (-/-). Plums: Cherry Plum-CP (+/-), Plum-P 620 (-/+), Ersinger-E (-/-), Italian plum-IP (+/+). (+/-) indicates level of carotenoids and polyphenols, respectively. B+S and B+S+D represents blank plus stimuli without and with digesta, respectively. No significant differences compared to positive controls (B+S+D=blank + stimuli + empty digests, which were set as 100%) were detected.

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Chapter 7: Proteomic Response of Inflammatory Stimulated Intestinal Epithelial Cells to *In Vitro* Digested Plums and Cabbages Rich in Carotenoids and Polyphenols

In this chapter 7, we have investigated the impact of carotenoids and polyphenols from plums and cabbages on the proteome of Caco-2 cells, a coculture of Caco-2/HT-29 MTX cells and THP-1 cells. The results were compiled in a manuscript entitled: Proteomic Response of Inflammatory Stimulated Intestinal Cells to *In-Vitro* Digested Plums and Cabbages Rich in Carotenoids and Polyphenols, Kaulmann, A., Planchon, S., Renaut, J., Schneider, Y.J., Hoffmann, L., Bohn, T., Food Funct, 2016, 7, pp 4388-4399.

Proteomic Response of Inflammatory Stimulated Intestinal Epithelial Cells to *In Vitro* Digested Plums and Cabbages Rich in Carotenoids and Polyphenols

Abstract:

Due to their anti-oxidant and anti-inflammatory potential, polyphenol and carotenoid-rich plant foods have been suggested as promising phytochemicals in the prevention of or as adjuvants regarding inflammatory bowel diseases (IBD). In the present study, we investigated whether plum (Italian Plum, *Prunus cocomilla*), or cabbage (Kale, *Brassica oleracea var. sabellica*), selected for their high phytochemical content, are able to reduce inflammation in cellular models of the intestinal epithelium, employing proteomic methods. For this purpose, plum/cabbage (carotenoid content: 2.0 mg per 100 g resp. 13 mg per 100 g; polyphenol content: 83 mg per 100 g resp. 27 mg per 100 g) were gastro-intestinally digested, and aliquots exposed (18 h) to either a monoculture (Caco-2) or a triple culture (Caco-2/HT-29-MTX (90 : 10, v/v) with THP-1 like macrophages), stimulated (with LPS, TNF-α, and IL-1β) to induce inflammation. Cells (Caco-2, Caco-2/HT-29-MTX, and THP-1) were then harvested separately, and proteomic analyses of total cell extracts were carried out by 2D-DIGE. In the monoculture, 68 protein-spots were significantly ($p < 0.05$, expression ratio >1.5) differentially regulated due to the Kale and Italian plum digesta, and in the co-culture 206 protein-spots, compared to digesta without plum/cabbage. These belonged to 27 (monoculture) and 76 (coculture) uniquely identified proteins, suggesting the coculture to be a more sensitive model. Proteins included antioxidant enzymes such as catalase, superoxide dismutase and glutathione-S-transferases. Only 3 proteins were differentially regulated in the THP-1 cells, perhaps as these were only indirectly exposed. The results show promise regarding some aspects

related to IBD complications, however, employing phytochemical-rich food items should be further investigated in *in vivo* trials.

7.1 Introduction

Inflammatory bowel diseases (IBD) describe a group of chronic relapsing inflammation complications of the gut. IBD frequently causes pain, vomiting, diarrhea, and can also increase the risk of developing colorectal cancer. Ulcerative colitis (UC) and Crohn's Disease (CD) are the most common IBDs.[1] In UC, the inflammation is localized mainly in the colon and the rectum, while in CD every part of the gastrointestinal tract can be affected. Their incidences have been increasing over the last years in Europe and in other Westernized Countries, to approx. 150–250/100 000 population.[2] It appears that they have a multifactorial etiology and that genetics, epigenetics and environmental factors play a role in the development of IBD.[2]

However, the change of dietary habits from frequent consumption of fish, vegetables and fruits to the intake of large amounts of sweetened beverages and foods, processed meats and fatty food appears to contribute to an enhanced risk.[3] It is also known that in the gut, many pro-oxidants may be produced through the plethora of microbes and their interaction with food components, in conjunction with immune cells, especially in diseased state. It has been emphasized that oxidative stress plays an important role in the onset and progression of IBD, and that in patients with IBD, plasma antioxidant capacity may be compromised,[2] though causes and effects are not always clearly separable, as also the inflammation of the epithelium can further aggravate oxidative stress.[2] As there is no real cure for IBD, one possible approach could be emphasizing preventive strategies, including diet. A limited number of non-traditional therapies against IBD have been advocated, including the intake of antioxidants and/or

anti-inflammatory functional foods, such as those rich in polyphenols.[4–8]

Carotenoids and polyphenols are phytochemicals which are known to possess both anti-oxidant and anti-inflammatory properties.[9–11] Several *in vitro* studies and animal trials have indicated that carotenoids and polyphenols are able to reduce inflammation, including that of the gastro-intestinal epithelium, possibly via interactions with different transcription factors such as the NF-κB or Nrf-2 pathway.[6,8] For example in a study by Romier *et al.*, the polyphenol chrysin, in a Caco-2 based model of the gut epithelium, was able to reduce NF-κB activation.[12] In cancer cells, the carotenoid lycopene showed to increase Nrf-2 translocation to the nucleus,[13] constituting an important transcription factor aiding in the activation of the bodies' own antioxidant enzymes, such as superoxide-dismutase (SOD), glutathione peroxidase (GPx) and heme-oxygenase 1 (HO-1).[14] Unfortunately, most of these studies have focussed on the administration of individual compounds, not on whole foods, without preceding digestion, and focussing on a rather limited number of targets.

In a previous study of ours, we have shown that digested plums and cabbages rich in carotenoids and phytochemicals were able to reduce some of the oxidative stress and inflammation related negative aspects in *in vitro* models of inflammation, which were partly related to NF-κB and Nrf-2 transcription.[15] Similar results, though with juice extracts, were shown in a previous study targeting anti-inflammatory aspects in human immune cells related to polyphenol-rich food items and altered gene-expression.[16] However, no

information is available on the influence of fruit and vegetable digesta regarding their effects on proteomic markers with respect to IBD, which would constitute a more global picture of potential health benefits than individual surrogate markers only.

In the present study, we aimed to investigate the effects of previously selected cabbage and plum digesta rich in carotenoids and polyphenols, on the proteome of epithelial cells as a simplified model of IBD. For this purpose, Caco-2 cells, and a co-culture of Caco-2 with HT-29 MTX (mucus producing) cells integrating also THP-1 macrophage-like cells, were pro-inflammatory stimulated (with TNF-α, IL-1β and LPS), and were successively exposed to digesta containing either Kale or Italian Plum, as an example of a vegetable and fruit rich in carotenoids and polyphenols.

7.2 Experimental

7.2.1 Chemicals

All products employed were of analytical grade or higher. Unless otherwise stated, all chemicals including interleukin-1β (IL-1β), lipopolysaccharide (LPS), arachidonic acid and resazurin, were procured from Sigma-Aldrich (St Louis, MO, USA). Penicillin/streptomycin mixture and non-essential amino acids were procured from Gibco (Karlsruhe, Germany) and Invitrogen (Camarillo, USA), respectively. Tumor necrosis factor-α (TNF-α) was procured from BD Biosciences (Erembodegem, Belgium). Milli-Q water (18.2 MΩ) was prepared with a purification system from Millipore (Brussels, Belgium) and used throughout.

from BD Biosciences (Erembodegem, Belgium).

7.2.2 Plum and Cabbage varieties and gastro-intestinal digestion

Cabbage and plum varieties were procured as described earlier.[17] In brief, 17 plum and 27 cabbage varieties were obtained from Luxembourgish farmers or supermarkets, and their macro- and micro-constituents, including phytochemicals, determined.[17] Varieties richest in polyphenols and carotenoids (Table 1) were retained for the present investigation, which were Kale (*Brassica oleracea var. sabellica* and Italian Plum (*Prunus cocomilla*)). Kale and plum were lyophilized (Christ freeze dryer, Thermo Fisher Scientific, Geel, Belgium) during 24 h, then homogenized with a grinder (Motor Grinder RM 200, Retsch, Aartselaar, Belgium) and stored in 50 mL centrifuge tubes at −20 °C until analysis. An *in vitro* digestion was carried out as described earlier,[18] simulating both the gastric and the small intestinal phase, containing the tested cultivars (0.5 g), and a certain amount of cream milk (3 g), required for assuring carotenoid solubilisation and bioaccessibility.[9]

Table 1: Native content of selected phytochemicals and macronutrients of Kale and Italian plum tested in this study (taken from Kaulmann *et al.* [70]).

	Native content in mg/100 g FW			Native content in g/100 g FW		Antioxidant test	
	Total carotenoids	Total polyphenols	Vitamin C	Dietary fibre	Total sugar	ABTS mg VCE/100g	FRAP µmol Fe(II)/100g
Kale	13	27	75.9	1.49	n.i.	190	1493
Italian plum	2.0	83	24.6	0.95	12.1	328	2264

Each value presents the mean of three replicate samples. FW = fresh weight. n.i. = no information. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). FRAP: Ferric ion reducing antioxidant power. VCE: vitamin C equivalents.

7.2.3 Cell cultures

The TC-7 subclone (ATCC Number: HTB-37) of the Caco-2 parental cell line was derived from a tumor isolated by J. Fogh [71], and generously donated by Monique Rousset (Nancy University, France). The HT-29-MTX cell line (ATCC Number: HTB-38) was a kind gift from Dr Técla Lesuffleur (Institut National de la Santé et de la Recherche Médicale, INSERM UMR S 938, Paris, France). Cultures were routinely maintained in 75 cm^2 flasks (Nunclon™, Nunc, Denmark) at 37°C and 10% CO_2 in a CB-210 CO_2 Incubator (Binder GmbH, Tuttlingen, Germany). Caco-2 and HT-29 MTX cells were grown in Dulbecco's modified eagle medium (DMEM + GlutaMAX (Gibco), supplemented with 1% nonessential amino acids, 10% heat-inactivated fetal bovine serum (Gibco), 1% of a mixture of penicillin and streptomycin (10,000 units and 10 mg mL^{-1}, respectively) and subcultured weekly after reaching ca. 80% confluence.

7.2.4 Cell viability

The reaszurin assay was used, to test the viability of the cells, especially following exposure to inflammatory stimuli and digesta [72]. Cells were exposed for 24 h to pro-inflammatory cytokines used to induce inflammation (25 ng mL^{-1} IL-1β together with 100 ng mL^{-1}

TNF-α and 10 μg mL^{-1} LPS) and to digesta (1:8, digesta: medium, v:v). After treatment, cells were washed with basal medium (DMEM + GlutaMAX, supplemented with 1% nonessential amino acids, 10% heat-inactivated fetal bovine serum (Gibco), 1% of a mixture of penicillin (10,000 units) and streptomycin (10 mg mL^{-1}), respectively) and incubated for 2 h with medium containing 400 μM resazurin in the dark (37°C, 90% air, 10% CO_2). Fluorescence was measured by a Synergy 2 plate-reader (BioTek, Colmar, France), with excitation at 530 nm (530±25 nm filter) and emission recorded at 590 nm (590±25 nm filter). Cell viability was expressed as mean cell viability (in %) compared to cells without treatment (set at 100%).

7.2.5 Exposure experiments

For the monoculture (Caco-2 cells) experiments, cells at passage numbers 70-72 and for the co-culture (Caco-2/HT-29 MTX cells) experiments, cells at passage numbers 65 to 71 for Caco-2 and 19 to 25 for HT29-MTX, were seeded on BD Falcon cell culture inserts (surface area 1 μm pore size, high pore density PET membranes for 6 well plates, BD Biosciences) at a cell density of 5 x 10^4 cells per cm^2, with 2 mL of basal media in the upper and lower compartment, 15

days before experiments. Five different treatments were performed (Table 2). To each well, 25 ng mL^{-1} of IL-1β, 100 ng mL^{-1} of TNF-α and 10 µg mL^{-1} LPS was added (hours 0-24 of exposure), with a total volume of 2 mL media (Fig. 1). Concentrations chosen were based on previous experiments [72] and additional reports [73]. Media contained aliquots of the respective digesta (hours 6-24) or not, diluted by 1:8 (Table 2).

For the co-culture (Caco-2/HT-29 MTX co-culture) experiment, cells at passage number 65 to 71 for Caco-2 and 19 to 25 for HT29-MTX were used. The co-culture part of the triple-culture (Caco-2, HT-29 MTX, 90:10 ratio) was seeded on BD Falcon cell culture inserts (surface area 1 µm pore size, high pore density PET membranes for 6 well plates, BD Biosciences) at a cell density of 5 x 10^4 cells per cm^2 and with 2 mL of basal media in the upper and 2 mL of basal media in the lower compartment, 15 days before experiments. One day before the experiments, THP-1 cells were seeded into new 6 well plates (BD Biosciences) at a cell density of 2.4 x 10^4 cells/cm^2 and differentiated with phorbol 12-myristate 13-acetate (PMA) into macrophage-like cells overnight in the incubator at 37°C and 5% CO_2 [74]. On the day of the experiment, media of THP-1 cells was removed and 2 mL of basal media was added, then the inserts with the co-culture part of the triple-

culture were also added. Five different treatments were performed (Table 2**)**. To each well, 100 ng mL^{-1} of TNF-α, 25 ng mL^{-1} of IL-1β and 10 µg mL^{-1} LPS was added (hours 0-24 of exposure) in the apical and the basolateral compartment (Fig. 1). Media in the apical compartment contained aliquots of the respective digesta (hours 6-24) or not, diluted by 1:8. Each experiment was performed on four different days with twelve replicates for each individual treatment.

Figure 1: General overview of the digestion and the experiment protocol.

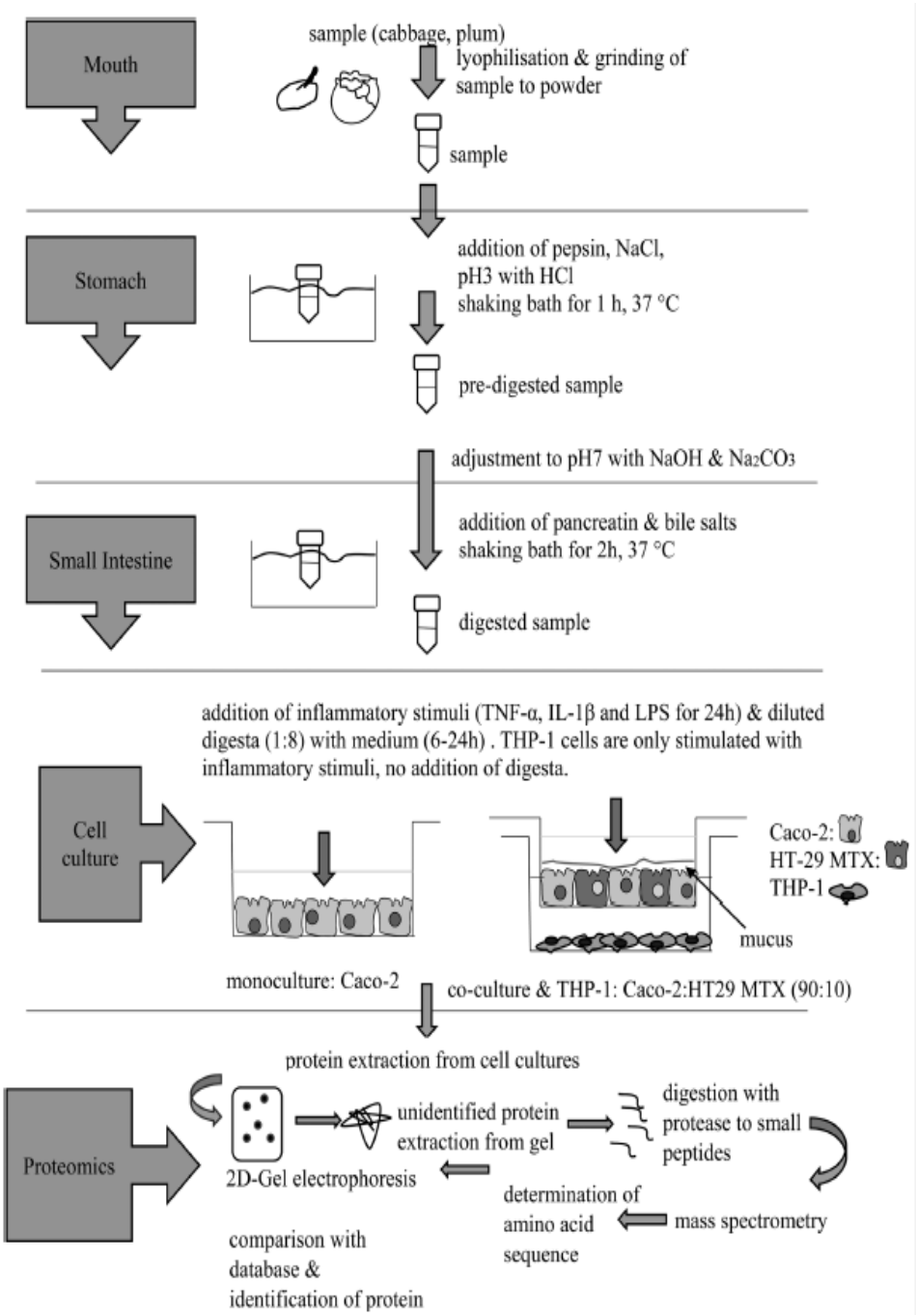


Table 2:
Exposure procedure of 15 day-differentiated Caco-2 cells and the triple-culture model (Caco-2/HT-29 MTX including 1 day differentiated THP-1 in the basolateral compartment) to various inflammatory stimuli and digesta (n= 12;N=4 per treatment).

Exposure group	Abbreviation	Treatment of cells[d]
Blank without stimuli	Blank	Untreated: no inflammatory stimuli or micelles
Blank with stimuli	B+S	Stimulation with TNF-α, IL-1β and LPS[b] (24h)
Stimuli with empty digesta[a]	S+ED	Stimulation with TNF-α, IL1-β and LPS[b] (24 h) and empty digesta (hours 6-24)
Stimuli with Kale[b] digesta	S+KA	Stimulation with TNF-α, IL1-β and LPS[b] (24 h) and digesta containing Kale (hours 6-24)
Stimuli with Italian plum[c] digesta	S+IP	Stimulation with TNF-α, IL1-β and LPS[b] (24 h) and digesta containing Italian plum (hours 6-24)

[a] Empty digesta: gastro-intestinal digesta carried out without the addition of plum or cabbage. [b]: 25 ng ml^{-1} IL-1β, 100 ng ml^{-1} TNF-α and 10 µg ml^{-1} LPS simultaneously to the apical compartment (Caco-2 cells) and apical and basolateral compartment alike (triple-culture). [c] Digesta (500 mg of dried matrix and 3 g cream milk) was diluted 1:8 with medium (v/v) before addition to the cells. [d] THP-1 cells in the basolateral compartment were only treated with stimuli, no addition of digesta to this compartment.

7.2.6 Treatment of cells and proteomic analyses following exposure of digesta

For 2D-DIGE, an earlier protocol [75] was adapted. Unless otherwise stated, all materials were from GE Healthcare (Uppsala, Sweden). 12 wells for each treatment (blank (B) – only media, blank+stimuli (B+S), stimuli+empty digesta (S+ED), stimuli+Kale digesta (S+KA), stimuli+Italian Plum digesta (S+IP)) were employed to obtain sufficient biological cellular material. Four biological replicates (N) were carried out on different days, each with 3 technical replicates (n), which were later pooled for proteomic analyses. After exposure treatments, cells were washed with 2 mL ice cold PBS and the different cells (Caco-2, Caco-2/HT-29, THP-1) were scraped off and transferred into a 15 mL Eppendorf tube. They were centrifuged at 4°C for 2 min at 200 g, then the supernatant was removed and cell pellets were frozen at -80°C until further analysis.

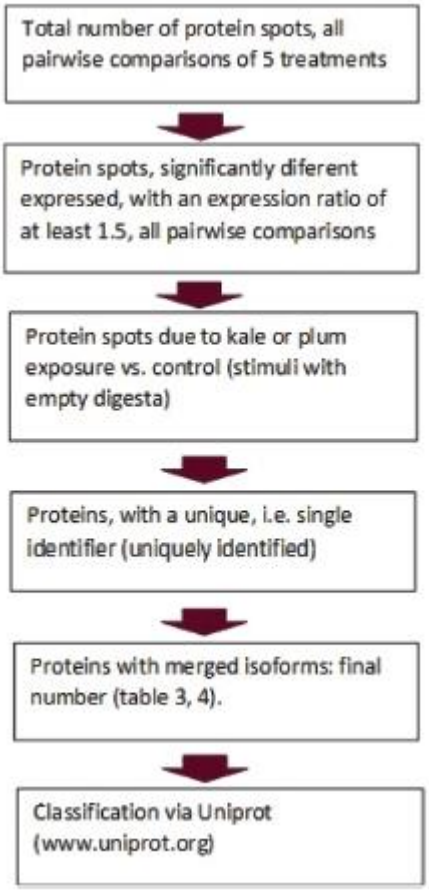
The pellets were solubilized with 200 µl of labelling buffer (7M Urea, 2M Thiourea, 30 mM Tris, 2% w/v CHAPS) on a thermomixer for 1 h at room temperature and 900 rpm. The samples were then filtered on an EASYstrainer™ 40 µm (Greiner Bio-one) by centrifugation at 3500 g for 5 minutes. The pH of the extracted protein samples was adjusted to 8.5 and the protein concentration was determined by the

Bradford method with BSA as a standard.[76] Each extract was then labelled by the minimal labelling process, following the manufacturer's instructions. In brief, 50 µg of proteins were labelled with either 400 pmol of Cy3 or Cy5 protein labelling dye, respectively. In addition, an internal standard was created by pooling aliquots of all protein extracts obtained, and 50 µg labelled with Cy2 dye. For the labelling, 400 pmol of dye were added to each tube, briefly centrifuged and vortexed and incubated for 30 min on ice in the dark. To stop the labelling reaction, 1 µl of 10 mM lysine was added; samples were then briefly centrifuged, vortexed and incubated for 10 min on ice in the dark. Afterwards, the Cy3 and Cy5-labelled extracts were combined with the Cy2-labelled internal standard. The volume was adjusted to 150 µl with sample buffer (urea (7 M), thio-urea (2 M), CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (0,5 %) and bromophenol-blue (traces)), then 9 µl of Servalyt™ 3-10 Iso-Dalt (Serva, Heidelberg, Germany) were added. A total of 150 µg proteins (50 µg per sample and 50 µg of internal sample) were then cup-loaded on each strip (Immobiline™ Drystrip pH 3-10 NL, 24 cm). Proteins were then subjected to isoelectric focusing on an IPGphor III at 20°C. The voltage was stepwise increased from 150 to 10,000 V during the first 16 h and then

stabilized at 10,000 V, until about 90 kVh were reached. The second dimension was carried out on an HPE™-FlatTop Tower (Serva) with a 2D HPE™ Large Gel NF 12.5% kit, following manufacturer's instructions. The gels were scanned using a Typhoon FLA 9500 scanner and analyzed by the DeCyder v.7.0. Selected spots of interest (abundance of variation 1.5 fold, $p<0.05$) were located on a gel and a "picking list" was generated. The spot picking was done with an Ettan spot picker (GE Healthcare), the digestion and the spotting of the samples on MALDI target was done automatically, using an EVO2 Workstation (TECAN). Peptide mass fingerprint and MS/MS analyses were carried out using the TOF/TOF 5800 System (AB sciex, CA). The ProteinPilot™ software 4.0.8085 was used for database searches with an in-house MASCOT platform (version 2.3, Matrix Science, www.matrixscience.com, London, UK). All proteins were identified by a search against the SWISSPROT database, downloaded in May 2015 and containing 548,454 sequences with a restriction to the Homo sapiens taxonomy (20,199 sequences). All were carried out using a mass window of 100 ppm for the precursor and 0.5 Da for the fragments. The following parameters were defined: two missed cleavages, fixed carbamido-methylation of cysteine, variable

oxidation of methionine or tryptophan, and tryptophan to kynurenine or double oxidation to N-formylkynurenine.

Figure 2: Procedure for protein determination by 2-D-Dige.



7.2.7 Data interpretation

Unless otherwise stated, all values are given as mean ± standard deviation (SD). Spots were considered to be differentially expressed if expression ratios were at least ±1.5 fold compared to the stimuli with empty digesta (control), and if p-values were below 0.05 (t-test). Only proteins that fulfilled these conditions and had a single unique identifier were included for final evaluation (Fig. 2).

The significantly over-and underexpressed proteins were categorized according to their molecular function according to gene ontology.

7.3 Results

7.3.1 Carotenoid and polyphenol concentrations in native matrices and following digestion

In a previous study, we measured the concentrations of carotenoids and polyphenols in the Kale and Italian plum. Carotenoids and polyphenols were extracted and analysed as described earlier [70]. Kale and Italian plum contained 13.3 resp. 1.9 mg/100g carotenoids and 26.9 resp. 82.7 mg/100g polyphenols (Table 1). The Kale and Italian plum were also exposed to an *in vitro* digestion as described earlier [77], employing both the gastric and the small intestinal phases. The bioaccessibility of the carotenoids and polyphenols were 6.8 % resp.

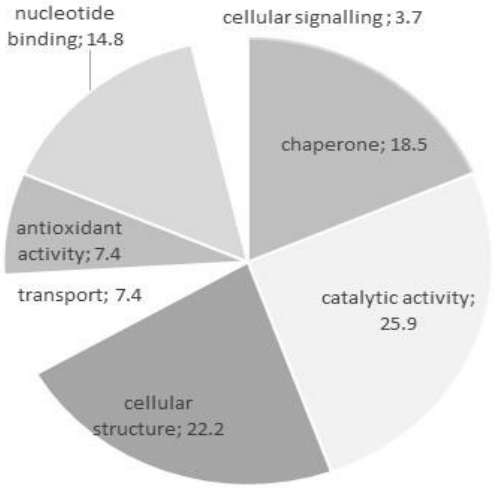
6.3 % for Kale and 3.4 % resp. 0.27 % for Italian plum. The uptake of carotenoids was 1-1.4% for Kale and 1-3% for Italian plum, depending on the cell culture used (monoculture vs. triple culture), while polyphenol uptake could not be clearly determined due to the manifold profile changes.

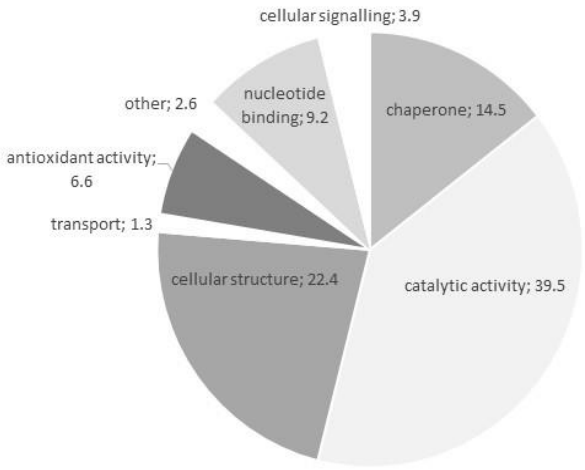
7.3.2 Proteomic results of the mono-culture

The analysis of the different treatments revealed that overall and within all pairwise comparisons (*i.e*. all 5 treatment groups compared against one another, with a total of 10 comparisons), 151 protein-spots were differentially expressed ($p < 0.05$, expression ratio at least 1.5 fold when compared to stimuli with empty digesta) in the monoculture. Out of these, 68 (also counting all isoforms) protein-spots were differentially expressed due to the Kale or Italian plum digesta, *i.e*. when comparing the groups "stimuli with blank" vs. "stimuli with kale" or "stimuli with Italian plum", as these were the comparisons of particular interest. Among these 68 differentially expressed protein-spots, 27 uniquely identifiable proteins (with isoforms merged) remained that were differentially expressed due to the Kale or Italian Plum digesta. From these 27 proteins, 25 were differentially expressed due to the Kale digesta and 8 due to the Italian

plum digesta (Table 3). The 8 proteins differentially expressed by the Italian plum digesta were mostly (6) downregulated compared to stimuli with empty digesta. For the Kale treatment, 7 proteins were upregulated and 15 proteins were downregulated. The 27 proteins were then classified according to their molecular functions, employing UNIPROT (http://www.unitprot.org/). Most of these proteins were involved in catalytic activity (26 %) and cellular structure (22%), Fig. 3.

Figure 3: Overview of protein molecular functions of the differentially expressed proteins following the various exposure experiments, defined by Gene Ontology. A: Caco-2. B: Co-culture.





7.3.3 Proteomic results of triple-culture

For the triple-culture, 340 protein-spots were differentially expressed ($p < 0.05$, expression ratio at least 1.5 fold compared to stimuli with empty digesta) in the co-culture part when considering all pairwise comparisons of exposure groups. Out of these, 206 protein-spots were differentially expressed due to the Kale or Italian plum digesta, *i.e*. when comparing the groups "stimuli with blank" vs. "stimuli with kale" or "stimuli with Italian plum". Among these 206 differentially expressed protein-spots, 76 uniquely identificable proteins (with isoforms merged) remained that were differentially expressed due to the Kale or Italian Plum digesta. Out of these, 73 proteins were differentially expressed due to the Kale digesta and 8 due to the Italian plum digesta (Table 4). The majority of proteins was downregulated compared to the control condition (stimuli with empty digesta). For the Kale treatment, 60 proteins were downregulated and 7 proteins were upregulated, 6 were both up-and downregulated (*i.e*. some isoforms up-, other downregulated). Most of the proteins were involved in catalytic activity (40%) and cellular structure (22%), Fig. 3.

Also following the plum treatment the majority of proteins (6) were downregulated, compared to the control.

For the THP-1 cells of the triple culture, 94 protein-spots were differentially expressed ($p < 0.05$, expression ratio at least 1.5 fold, compared to stimuli with empty digesta) when considering all pairwise comparisons of exposure groups. Out of these, only 3 proteins were differentially expressed due to the Kale or Italian plum treatment, *i.e*. comparing "stimuli with blank" vs. "stimuli with kale" or "stimuli with Italian plum". However, only one of these proteins (serine/arginine-rich splicing factor 9, which was significantly downregulated by kale) could be uniquely identified and was considered not likely to be related to IBD disease prevention or treatment.

Table 3: List of differentially expressed and uniquely identifiable proteins[a] following Caco-2 cell (monoculture) exposure with digested plum and cabbage, compared to empty digesta (digestion without plum or cabbage variety but also with stimuli).

Protein count	Protein name	Activity Category	Protein class	Kale vs. control	Italien Plum vs. control
1	3-mercaptopyruvate sulfurtransferase	Antioxidant activity	Transferase		
2	60 kDa heat shock protein, mitochondrial	Chaperone	Protein assembley		
3	78 kDa glucose-regulated protein	Chaperone	Protein folding		
4	Aconitate hydratase, mitochondrial	Catalytic activity	Lyase		
5	ATPase inhibitor, mitochondrial	Catalytic activity	Ligase		
6	Cadherin-17	Transport	Cell junction protein		
7	Catalase	Antioxidant activity	Peroxidase activity		
8	Desmoglein-2	Transport	Cell junction protein		
9	Endoplasmin	Chaperone	Protein processing		
10	Ezrin	Cellular structure	Actin binding		
11	Heat shock protein 75 kDa, mitochondrial	Chaperone	Protein assembley		
12	Heterogeneous nuclear ribonucleoprotein K	Nucleotide binding	RNA binding		
13	Histone H2B type 1-M	Nucleotide binding	DNA binding		
14	Keratin, type II cytoskeletal 8	Cellular structure	Filament organization		
15	Meprin A subunit alpha	Catalytic activity	Hydrolase		
16	MIC complex subunit MIC60	Cellular structure	Membrane architecture		
17	Neutral alpha-glucosidase	Catalytic activity	Hydrolase		
18	Nucleolin	Nucleotide binding	DNA binding, chromatin decondensation		
19	Prelamin-A/C	Cellular structure	Structural protein		
20	Prohibitin	Nucleotide binding	DNA proliferation		
21	Prolyl 4-hydroxylase subunit alpha-1	Catalytic activity	Oxidoreductase, collagen synthesis		
22	Protein disulfide-isomerase A3	Catalytic activity	Cell redox homeostasis		
23	Protein S100-A10	Cellular signalling	Protein phsophorylation		
24	Pyruvate kinase PKM	Catalytic activity	Kinase, ATP formation		
25	Stress-70 protein, mitochondrial	Chaperone	Protein assembley		
26	Tubulin beta-4B chain	Cellular structure	Microtubili		
27	Villin-1	Cellular structure	Actin binding		

No change
Upregulated
Downregulated
Generally upregulated (some isoforms upregulated, others unchanged)
Generally downregulated (some isoforms downregulated, others unchanged)
Up-and downregulated (some isoforms upregulated, others downregulated)

Compared to control (stimuli with empty digesta) [a]Under the condition of $p<0.05$ (t-test) and expression ratio ±1.5 compared to stimuli with empty digesta.

Table 4: List of differentially expressed and uniquely identifiable proteins[a] following triple cell culture exposure (co-culture part harvested) with digested plum and cabbage, compared to empty digesta (digestion without plum or cabbage variety but also with stimuli).

Protein count	Protein name	Activity Category	Protein class	Kale vs. control	Italian Plum vs. control
1	14-3-3 protein beta/alpha	Chaperone	Protein assembley		
2	14-3-3 protein zeta/delta	Chaperone	Protein assembley		
3	2-oxoglutarate dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
4	3-ketoacyl-CoA thiolase, mitochondrial	Catalytic activity	Acetyltransferase		
5	60 kDa heat shock protein, mitochondrial	Chaperone	Protein assembley		
6	78 kDa glucose-regulated protein	Chaperone	Protein assembley		
7	Acetyl-CoA acetyltransferase, mitochondrial	Catalytic activity	Transferase		
8	Aconitate hydratase, mitochondrial	Catalytic activity	Lyase		
9	Adenylate kinase 4, mitochondrial	Catalytic activity	Nucleotide kinase		
10	Aldehyde dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
11	Annexin A2	Other	Calcium binding, cell motility		
12	Annexin A5	Other	Antiocoagulant, calcium-binding		
13	ATP synthase subunit alpha, mitochondrial	Catalytic activity	ATP synthesis		
14	ATP synthase subunit beta, mitochondrial	Catalytic activity	ATP synthesis		
15	ATPase inhibitor, mitochondrial	Catalytic activity	Ligase		
16	Catalase	Antioxidant activity	Peroxidase activity		
17	Cathepsin D	Catalytic activity	Hydrolase		
18	Cofilin-1	Cellular structure	Actin binding		
19	Complex I intermediate-associated protein 30, mitochondrial	Chaperone	Protein assembley		
20	Creatine kinase B-type	Catalytic activity	Transferase		
21	Cytochrome c oxidase subunit 5A, mitochondrial	Catalytic activity	Electron transport		
22	DAZ-associated protein 1	Nucleotide binding	RNA binding		
23	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	Catalytic activity	Beta-oxidation, isomerase		
24	Electron transfer flavoprotein subunit beta	Catalytic activity	Electron transport		
25	Endoplasmin	Chaperone	Protein assembley		
26	Enoyl-CoA delta isomerase 1, mitochondrial	Catalytic activity	Isomerase		

Table 4: Continued

Protein count	Protein name	Activity Category	Protein class	Kale vs. control	Italian Plum vs. control
27	Enoyl-CoA hydratase, mitochondrial	Catalytic activity	Lyase		
28	Eukaryotic initiation factor 4A-III	Cellular structure	RNA helicase		
29	Ezrin	Cellular structure	Actin binding		
30	Galectin-3	Cellular signalling	Galactose binding		
31	Gelsolin	Cellular structure	Actin modulating protein		
32	Glutamate dehydrogenase 1, mitochondrial	Catalytic activity	Oxidoreductase		
33	Glutathione S-transferase A1	Antioxidant activity	Transferase		
34	Glutathione S-transferase kappa 1	Antioxidant activity	Transferase		
35	Glutathione S-transferase P	Antioxidant activity	Transferase		
36	Heat shock protein 75 kDa, mitochondrial	Chaperone	Protein assembley		
37	Heat shock protein HSP 90-beta	Chaperone	Protein assembley		
38	Heterogeneous nuclear ribonucleoprotein A/B	Nucleotide binding	mRNA binding		
39	Heterogeneous nuclear ribonucleoprotein H	Nucleotide binding	mRNA binding		
40	Heterogeneous nuclear ribonucleoprotein M	Nucleotide binding	mRNA binding		
41	Heterogeneous nuclear ribonucleoprotein U-like protein 2	Nucleotide binding	RNA binding		
42	Heterogeneous nuclear ribonucleoproteins A2/B1	Nucleotide binding	mRNA binding		
43	Keratin, type I cytkeletal 18	Cellular structure	Filament organization		
44	Keratin, type I cytkeletal 19	Cellular structure	Filament organization		
45	Keratin, type II cytkeletal 8	Cellular structure	Filament organization		
46	Malate dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
47	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
48	MIC complex subunit MIC60	Cellular structure	Membrane architecture		
49	Moesin	Cellular structure	Cytoskeleton, cell adhesion		
50	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	Catalytic activity	Oxidoreductase		
51	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	Catalytic activity	Oxidoreductase		
52	Nucleophmin	Chaperone	Protein assembley		
53	Ornithine aminotransferase, mitochondrial	Catalytic activity	transferase		
54	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	Catalytic activity	Lyase		
55	Phosphoglycerate mutase 1	Catalytic activity	Isomerase		
56	Polyubiquitin-B	Cellular structure	Structural protein		
57	Prelamin-A/C	Cellular structure	Structural protein		
58	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	Cellular structure	Collagen formation		
59	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3	Cellular structure	Collagen formation		

Table 4: Continued

Protein count	Protein name	Activity Category	Protein class	Kale vs. vs. control	Italian Plum vs. control
60	Protein disulfide-isomerase	Catalytic activity	Cell redox homeostasis		
61	Protein disulfide-isomerase A3	Catalytic activity	Cell redox homeostasis		
62	Protein NDRG1	Cellular signalling	Cell growth and differentiation		
63	Protein S100-A10	Cellular signalling	Protein phosphorylization		
64	Pyrroline-5-carboxylate reductase 1, mitochondrial	Catalytic activity	Oxidoreductase		
65	Radixin	Cellular structure	Actin binding		
66	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
67	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
68	Stress-70 protein, mitochondrial	Chaperone	Protein assembley		
69	Superoxide dismutase [Mn], mitochondrial	Antioxidant activity	Oxidoreductase		
70	TATA-binding protein-associated factor 2N	Nucleotide binding	DNA binding		
71	T-complex protein 1 subunit zeta	Chaperone	Protein assembley		
72	Tubulin alpha-1B chain	Cellular structure	Microtubili		
73	Tubulin beta-4B chain	Cellular structure	Microtubili		
74	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	Catalytic activity	Oxidoreductase		
75	Villin-1	Cellular structure	Actin-binding		
76	Voltage-dependent anion-selective channel protein 2	Transport	Anion-channel		

No change
Upregulated
Downregulated
Generally upregulated (some isoforms upregulated, others unchanged)
Generally downregulated (some isoforms downregulated, others unchanged)
Up-and downregulated (some isoforms upregulated, others downregulated)

Compared to control (stimuli with empty digesta) [a]Under the condition of $p<0.05$ (t-test) and expression ratio ± 1.5 compared to stimuli with empty digesta

7. 4 Discussion

In this study, we investigated the hypothesis that carotenoids and polyphenols from plums and cabbages, following simulated gastro-intestinal digestion, might show beneficial effects on protein expression regarding aspects of IBD, such as proteins involved in anti-oxidant and anti-inflammatory effects on cellular models of the intestinal epithelium. To our knowledge, this is the first proteomic study investigating the effects of digested phytochemical-rich foods in an *in vitro* model of IBD. For this purpose, we tested the influence of Italian plum and Kale digesta, selected for being rich in both carotenoids (1.9 resp. 13.3 mg per 100 g) and polyphenols (82.7 resp. 26.9 mg per 100 g), on the proteome of inflammatory stimulated Caco-2 cells and on the proteome of an inflammatory stimulated triple-culture, consisting of a Caco-2/ HT-29 MTX co-culture and THP-1 cells, better simulating effects of the immune system.[15] We expected to see effects, as previously targeted investigations showed positive effects of these varieties, including the reduction of certain cytokines, in conjunction with reduced expression of NF-κB and reduced Nrf-2, in line with an overall reduction of inflammation and oxidative stress, respectively.

The results revealed that in the Caco-2 monoculture a total of 27 proteins were differentially expressed by the Italian plum and Kale digesta exposure when compared to digesta without fruit or vegetable (empty digesta), while in the co-culture of the triple cell model, 76 proteins were differentially expressed, suggesting that the co-culture may be a more responsive model for investigating inflammatory aspects, even though in the THP-1 cells themselves, only one protein

was differentially expressed following the kale/plum treatments. The higher response of the co-culture as opposed to the monoculture was already observed in a former study, where the effects of the same Italian plum and Kale digesta on transcriptomic responses and targeted markers was studied.[15] The different plum and cabbage digesta showed a slightly higher response regarding IL-8 and NO secretion, as well as on the expression level and nucleus accumulation of Nrf-2 in the co-culture compared to the monoculture. This phenomenon may be explained by a possibly higher cellular uptake of the carotenoids in the co-culture, as also reported earlier,[18] perhaps as the mucus produced from the HT-29 MTX cells may aid in the binding of the mixed micelles to the cell, enhancing carotenoid uptake. Another possibility, though perhaps less likely, is that the interchange of signals between the apical layer (Caco-2/HT-29 MTX cells) with the basolateral macrophage-like cells (THP-1) resulted in an enhanced stimulus of the apical layer, even though no strong response was observed in the THP-1 cells themselves. This reaction of the THP-1 cells to the treatment may have been expected, as the THP-1 cells were not in direct contact with the digesta (see Fig. 1), and were thus exposed to lower concentrations of digesta, and exposure time may have been too short for more pronounced effects. Though in the previous transcriptomic study (performed following the same conditions), THP-1 cells (following kale exposure) showed diminished IL-6 and NF-κB activation, it is possible that the present incubation time of 18 h with the digesta was too short to produce a full response at the proteomic level.

The treatment with the Kale digesta showed also a much more pronounced response regarding the number of differentially expressed proteins than the treatment with Italian plum digesta (25 and 73 for kale in the mono- and triple-culture, respectively, compared to both 8 in the mono- and triple culture for Italian plum), in both cell-models. We can only speculate for the underlying reasons. This result would be in line with a higher amount of carotenoids in Kale compared to the tested plum (and with a similar fractional absorption for Kale and Italian plum, *i.e*. 1% for the monoculture and 3% for the co-culture).[18] It can also not be excluded that other components of the cabbage, such as glucosinolates, *e.g*. glucoraphanin, known for their anti-apoptotic properties,[24] have contributed to the observed effects. The vitamin C content was also higher in Kale compared to the tested plum (76 mg per 100 g *vs*. 25 mg per 100 g).

The significantly over-or underexpressed proteins were, to a certain extent, involved in oxidative stress. The proteins in the co-culture part of the triple cell model were mostly downregulated (79%), which is in line with the proteins of the monoculture, of which 56% were downregulated. Several of the differentially regulated proteins were enzymes, among other catalase, glutathione S-transferase, and SOD, all involved in antioxidant defence mechanisms (Tables 3 and 4). Increased oxidative stress can lead to activation of the inflammasome, *e.g. via* stimulating macrophages or other immune cells, following oxidative burst.[25] Catalase and SOD are among the most important antioxidant enzymes of the human defence mechanism.[26] SOD, down-regulated in the co-culture in the present study, converts the superoxide anion (O^{2-}) into hydrogen peroxide

(H_2O_2), and catalase, down-regulated in both our cellular models, then catalyses the decomposition of H_2O_2 into water and molecular oxygen.[27] These two antioxidant enzymes play a key role in maintaining the homeostasis of pro-oxidants and anti-oxidants. They also play a role in the protection against several pathological conditions. In a study done by Shi *et al.*, the administration of recombinant catalase to influenza A infected mice, resulted in a decrease of different inflammatory cytokines *e.g.* IL-6.[28] Similarly, the administration of a melon concentrate rich in SOD during one month to obese hamsters, significantly decreased IL-6 secretion by 45%, TNF-α by 28% and NF-κB activation by 5% in the liver.[29]

Peroxiredoxins are another class of enzymes that degrade hydroperoxides to water, thus constituting crucial members of the antioxidant defence mechanism.[30] A study on mouse embryonic fibroblasts derived from peroxiredoxin-I knockout mice showed that this knockout increased the expression of IkB, TNF-α and IL-6 and down-regulated the gene expression of Nrf-2 and hemeoxygenase-1, following irradiation with UVA.[31] In the present study, peroxiredoxin-4 could not be uniquely identified, but was suggested to be down-regulated for the co-culture and the monoculture, which would be in line with the down-regulation of the other antioxidant enzymes. Glutathione-*S*-transferases (GST), down-regulated in the coculture in the present study, are a large and diverse group of enzymes, responsible for the detoxification of xenobiotics and by-products of oxidative stress.[32] In a study with LPS-stimulated mouse lung alveolar epithelial cells (C-10 cell line), knocking down glutathione *S*-transferase P (GSTP) increased NF-κB activation and pro-oxidant

cytokine production.[33] In the co-culture, the Kale treatment decreased various types of glutathtione-*S*-transferases, following the same pattern as observed for catalase and SOD.

As in the co-culture, these antioxidant enzymes were downregulated by especially the Kale treatment also in the monoculture. This could lead to the hypothesis that both carotenoids and polyphenols of the Kale reduced the general redox and inflammatory state of the cells, resulting in down-regulation of antioxidant related enzymes. These finding are in line with the earlier transcriptomic study, were Kale significantly reduced the Nrf-2 nuclear accumulation in the co-culture, an important transcription factor maintaining the physiological level of reactive oxygen species.[15] Similarly, lower but physiological levels of antioxidants have been shown to likewise reduce the translocation of Nrf-2. For example, in a study with trans-retinoic acid, lower physiological levels reduced translocation of Nrf-2 to the nucleus, while higher, presumably toxic levels enhanced Nrf-2 translocation,[6,15] emphasizing dose-depending response effects. However, as in the present study many catalytic activities were generally down-regulated, it cannot be excluded that the kale and plum treatment reflected a general slowing down of the cellular metabolism following these treatments.

Regarding anti-inflammatory markers, as found to be in part down-regulated in our previous transcriptomic study,[15] these could not be clearly identified in the present study. It is generally expected that decreased oxidative stress would go along with decreased levels of inflammation, also in IBD.[6,8] However, several of the heat-shock proteins that were mostly down-regulated in the present study,

involved in cell proliferation and cellular ageing, have been reported to influence cytokine expression such as interleukins (pro-as well as anti-inflammatory ones), and thus may reflect the involvement of inflammation related aspects.[34,35] Only very few studies have looked at proteomic effects of carotenoids. A study employing lycopene and MCF-7 (breast cancer cell line) and MCF-10 (non-tumorigenic epithelial breast cell line) cells showed that lycopene had a different impact on the proteome of the two cell lines. In MCF-7 cells, lycopene inhibited cell proliferation, while in MCF-10 cells it did not show an effect.[36] Another study with lycopene and human primary prostate epithelial cells showed that lycopene up-regulated glutathione-*S*-transferase, and other anti-oxidant enzymes.[37] In a former study of ours, we investigated the impact of lycopene and beta-carotene in artificial micelles on the proteome of Caco-2 cells, showing that beta-carotene had a higher impact on the Caco-2 proteome than lycopene (16 differentially expressed proteins *vs.* 1 differentially expressed protein). Most of these proteins differentially expressed by beta-carotene were down-regulated and involved in oxidative stress, such as glutathione-*S*-transferase,[19] which is in line with results of the present study, though the absence of digestion and altered bioavailability from artificial micelles could have created differences in the strengths of response. We also have to point out limitations of this study. We exposed the monoculture and the co-culture part of the triple cell model to the digesta for 18 hours, which was perhaps not long enough to spot interactions between the co-culture part and the THP-1 cells of the triple model, as indicted by the relatively low proteomic response of the THP-1 cells. However,

longer exposure also increases the risk of bacterial infections *via* the added digesta. Furthermore, anti-oxidant status in the cells was not measured in this study or the previous experiments.

7.5 Conclusions

In summary, we investigated the effect of Kale and Italian plum digesta on the proteomic response of two different cell models of intestinal inflammation: a Caco-2 cell mono-culture and a triple-culture comprised of a Caco-2/HT-29 co-culture and THP-1 cells. The results highlight that the choice of model, *i.e*. with and without mucus and responding immune cells (the latter one being more physiological), perhaps related to the uptake of antioxidants, may results in different strengths of response. The Kale digesta showed a high impact on different important antioxidant enzymes, *e.g*. catalase, glutathione transferase, and SOD in the co-culture part of the triple cell model and catalase in the monoculture, in line with an overall reduction of oxidative stress by the Kale and Italian Plum exposure. Further investigations with longer exposure times and other varieties of Kale and Plum are warranted.

Conflict of interest

The authors declare no conflict of interest.

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8. Conclusions (Chapter 8):

Chosen varieties, native polyphenol and carotenoid content

Several nutrition and health related organizations recommend a diet rich in fruit and vegetables. Fruits and vegetables contain a large array of nutrients and phytochemicals with health-related properties and are known to reduce the risk of several chronic diseases, among others, inflammatory bowel disease (IBD) [1]. However, it is until now poorly understood which of these nutrients and phytochemicals are the most bioactive. Carotenoids and polyphenols present the two most important classes regarding lipid and water-soluble phytochemicals, respectively, and they are known to possess anti-oxidative and anti-inflammatory properties [2-4]. A number of studies investigated the relation between the dietary intake of carotenoids or polyphenols and chronic diseases, such as diabetes or cardiovascular disease, but there is limited information about the effect on IBD. The most predominant IBD are Crohn´s disease (CD) and ulcerative colitis (UC), which are both characterized by the destruction of the gastrointestinal mucosa, and which can lead in the worst case to cancer. In Europe and North America, 0.4 % of the population suffer from IBD [5], with clearly increasing incidence over the last decades. As IBDs are virtually uncurable, prevention and amelioration strategies to strengthening the gastro-intestinal mucosa integrity are crucial. These strategies include choosing food items rich in bioactive phytochemicals with anti-oxidant and anti-inflammatory properties, as the progression of IBD is clearly related to local and systemic deterioration of subjects' anti-oxidant and anti-inflammatory status [6].

In this study, we first focused on the characterization of different plum and cabbage varieties, as these two food crops are frequently consumed in Luxembourg, with intakes of *ca*. 16 kg/year and 13.8 kg/year, and are rich in carotenoids and polyphenols (2 and 30-60 mg/100g repectively in Kale and 0.4 and 115 mg/100g, respectively for plum). We speculated that certain varieties of plum and cabbage would be especially rich in carotenoids and polyphenols. We also wanted to see how the profile of the carotenoids and polyphenols is changing during gastro-intestinal digestion and different kitchen procedures, how bioavailable carotenoids and polyphenols were from the different food matrices and how they would affect inflammatory responses. This study should thus give us an improved understanding of how carotenoids and polyphenols affect molecular pathways that are involved in inflammatory processes.

The first step was therefore to determine the carotenoid and polyphenol content, as well as selected micro- and macro-nutrient profiles of 17 different plum varieties and 27 different cabbage varieties grown in Luxembourg. We observed that there was a high variability of carotenoid and polyphenol content between the different plum and cabbage varieties and that cabbage varieties contained higher carotenoid concentration than plum varieties, while plum varieties contained higher polyphenol concentrations than cabbages. The highest carotenoid content was found in Kale with 13.3 mg/100g and in Cherry and Italian plums with 2.0 and 1.9 mg/100g, respectively [7]. The highest polyphenol content was found in Kale with 26.9 mg/100g and in Kirks' plum with 152 mg/100g. The consumption of cabbage and plum was estimated to contribute to the

daily intake of 26, respectively 6 mg ascorbic acid equivalents. The results suggest a high inter-individual variability of phytochemicals between the various cultivars, possibly related to genetic but also environmental aspects such as climate and soil conditions. As our studies were targeted at investigating only the scope of variation, partly in order to choose the most suitable varieties for follow-up studies, we did not attempt to determine via multi-seasonal measurements the individual contribution of genetic *vs.* environmental aspects [8, 9].

By multivariate regression analyses, we also strived to determine which of the tested phytochemicals and micro- and macronutrients contributed the most to the anti-oxidant capacity, as measured *in vitro*. Phenolic constituents and ascorbic acid and, for cabbage varieties, also lutein, for plum varieties, also total sugar and selenium, predicted the best anti-oxidant capacity. Total sugar has shown in some studies a positive correlation with total phenolic content, probably as it plays a role in phenolic biosynthesis.[10, 11] This suggested that not only one single component may best predict anti-oxidant capacity, but that there are additive/synergistic effects of the different components. It has, however, to be stated that carotenoid contribution to anti-oxidant capacity could have been underestimated as the FRAP and ABTS tests had limited extraction capacity for lipophilic constituents. It is also difficult to predict, based on these *in vitro* results, any bioactivity *in vivo*, as *in vivo* both the carotenoids and polyphenols are cleaved or metabolized into compounds with altered bioactivity compared to the parental molecules, in addition to limited bioavailability of some

carotenoids/polyphenols. However, antioxidant activity may constitute a good measure of the presence of bioactive phytochemicals [12].

Another group of compounds that was measured includes dietary fibre, which may also have prebiotic functions, possibly also improving gut–health [13]. In our varieties, the dietary fibre content was higher in cabbage (1-7g/100g) than in plum varieties (0.47 -1.3 g/100g). The highest dietary fiber content was found in Kale (7g/100g) and in plum 620 (1.3 g/100g).

Though many changes occur following ingestion and digestion regarding the profile of carotenoids and polyphenols, generally reducing antioxidant capacity *in vivo* [14], it is possible that these compounds are able to quench free radicals in the gut, *i.e.* prior to their absorption, and this may also result in health beneficial effects, including the reduction of IBD risk, though there is, to our knowledge, no research published on this topic.

Some limitations of our investigations can also be pointed out: for example, we used the whole fruit and vegetable matrix and we saw that carotenoids and polyphenols only constitute ± 2% of the whole dry matrix (Table 1).

Table 1: Measured dry matrix composition of cabbage and plum

	Cabbage	Plum
Dietary fibre	79 %	4.7 %
Vitamin C	2%	0.2 %
Total Mineral content	14%	1.2 %
Sugar	Not measured	83%
Titratable acidity	2.9 %	8.9 %
Total carotenoid & polyphenols	2.3 %	1.8 %

The limitation of using whole fruits and vegetables is that one cannot attribute the anti-oxidant capacity to one single phytochemical, as we noticed by the multivariate regression analyses. Some studies showed that the consumption of whole fruits and vegetables reduced the risk of chronic diseases, however the consumption of single components, such as β-carotene for example, did not reduce the risk or even aggravated the disease [15]. Some studies also focused on the synergistic effects between different phytochemicals. Liu *et al*. [16] measured the scavenging capacity of lycopene, vitamin E, vitamin C and β-carotene alone and as mixtures, with the help of the DDPH method and found out that the mixture containing all four compounds showed the highest scavenging capacity. Another study, done by Dai *et al.* [17], showed that the mixture of green tea polyphenols, vitamin E and vitamin C acted synergistically on the reduction of lipid peroxidation, as studied in sodium dodecyl sulphate (SDS) micelles.

Another limitation of our investigations is that we did not measure other anti-oxidant compounds such as glucosinolate, tannins or triterpenoids, which also have been attributed to certain health effects.

Glucosinolates for example showed some effect on anti-cancer progression [18], but less is known on their anti-oxidant and anti-inflammatory potential [19]. Tannins are known for their cardioprotective effects [20], but they also known for their anti-oxidant properties [21 22]. Triterpenoids are biologically active compounds found in high concentrations in fruit cuticular waxes. Prunes together with apple, cherry and pear are the richest natural sources for ursolic acid [23]. Triterpenoids of apple peel (mostly ursolic acid) showed for example antitumor activity in HL-60 cells [24].

Influence of kitchen procedures

Many of the kale varieties are not consumed in raw form but following various cooking procedure. It is known that the original carotenoid and polyphenol composition of the plums and cabbage varieties undergo changes during kitchen procedures, gastro-intestinal digestion, and colonic fermentation [14, 25-29] and in order to better assess their bioactivity *in vivo* such changes should be estimated.

For this purpose, we used Kale as a model and either we froze and defroze the Kale or we chopped the Kale and then steamed, boiled or froze the chopped Kale. Carotenoids were not significantly impacted *i.e* reduced by the selected kitchen procedures, while polyphenols were significantly impacted by chopping and heating (boil or steam), by up to 89 and 51 % respectively. We have to state that we did not subject plums to any kitchen procedures, such as occurring during jam fabrication and baking into pies, as plums are relatively often eaten raw.

It is known that maceration and the implication of heat can increase the availability of carotenoid and polyphenols [30, 31], but on the other hand if carotenoids and polyphenols are released from their matrix they can also be degraded by the heat, which we saw in our study for the polyphenols. The higher degradation of polyphenols compared to the carotenoids could also be explained by additional degradation by enzymes e.g polyphenol oxidase. The implication of hot water on the Kale, during boiling, showed the worst impact on polyphenol availability while freeze/defreezing seemed to have only a slight impact.

It has to be stated that during our study, we did not use the cooked Kale for *in-vitro* gastro-intestinal digestion and further carotenoid and polyphenol measurement for bioavailability and cellular uptake. This could have been of interest, as the bioavailability of carotenoids from cooked meals, is often higher than that carotenoids from raw meals. A study done with a raw carrot meal vs., a cooked puree carrot meal, showed that the bioavailability of β-carotene from cooked puree carrot meal was higher compared to the raw carrot meal (65 % vs 41%) [32]. Bugianesi *et al.* showed that plasma concentration of naringenin and chlorogenic acid significantly increased after ingestion of cooked cherry tomatoes, but not after ingestion of raw cherry tomatoes [33].

Bioaccessibility and cellular uptake of carotenoids and polyphenols

Following the determination of carotenoid and polyphenol profile of plums and cabbages, we investigated how the profile changed during simulated digestion, determining the fraction available for absorption. For this purpose, we submitted four plum (Italian plum, Ersinger, Plum 620 and Cherry plum) and four cabbage varieties (Kale, Kalorama, Scots Kale and Duchy) with contrasting (*i.e.* either rich in carotenoids and poor in polyphenols, vice versa, or rich and poor in both) constituents to an *in vitro*-gastrointestinal digestion. The *in vitro*-gastrointestinal digestion employed both the gastric and small intestinal phases was described earlier [34]. The digesta were then also exposed to colonic fermentation, *i.e.* mixed with inocula prepared from pooled pig feces [35]. The carotenoid profile was not significantly impacted by the gastro-intestinal digestion but the polyphenol profile was dramatically influenced as most compounds were no longer detectable (probably due to very low concentration) with the UPLC method we used. This loss was possibly due to, at least in part, the addition of cream, which was included as a lipid source to facilitate carotenoid bioaccessibility. Earlier studies have shown that cream impaired the bioaccessibility of chlorogenic acids, being together with its isomer neochlorogenic acid the predominant polyphenols in our varieties, probably due to interaction of polyphenols with proteins [36]. Cream was chosen as a lipid source for the hydrophobic carotenoids, as it showed the best micellarization effects [37]. It has however also to be stated that a lot of other emulsifiers (lecithin and oleic acid) were used in these other studies, which could have facilitated the fat

dissolution in the digesta. Unfortunately, there are not many studies that have investigated the effect of vegetable lipid on polyphenol bioaccessibility and cellular uptake/absorption. Another important point for the low polyphenol bioaccessibility might also be that most of the polyphenols remain in the non-extractable fraction and that this fraction together with the re-excreted polyphenols undergoes then colonic fermentation. The non-extractable fraction of polyphenols represents the major part of dietary polyphenols and are often higher in content than extractable polyphenols[38]. The extraction method we used was based on methanol extraction, which is a good established extraction method [39], but we should keep in mind that not all polyphenols are extracted with this method. However, it is likewise assumed that these polyphenols would also not be extractable by the upper gastro-intestinal system, which was studied in the present investigation, though some non-extractable polyphenols may be released following colonic fermentation.

In our study, we saw that Kale had the highest bioaccessibility of total polyphenols with 6.8 %, the polyphenol content of the other three cabbage varieties was not detectable after digestion. In plums, the bioaccessibility was low and varied between 0.14 and 0.38 %.

Carotenoid bioaccessibility was slightly higher in plum varieties than in cabbage varieties (6.8% *vs.* 4.4%). It can only be speculated, but possibly, this could be related to the fact that, in plums, carotenoids are present in liquid-crystallised or dissolved in lipid droplet form and, in cabbages, in rather large crystalloid form, which could limit the transfer to the aqueous phase.

The cellular uptake of carotenoids and polyphenols was determined with the help of two different cell models, on one hand, monoculture of only Caco-2 cells and, on the other hand, coculture of Caco-2 and mucus producing HT-29-MTX cells. We did choose these two different cell models to see if the mucus produced by the HT-29 MTX cells might have an impact on the bioavailability of the carotenoids and polyphenols, as the influence of mucus has been discussed earlier as an additional barrier for the uptake of bioactive compounds such as polyphenols [40]. Caco-2 cells are a well representative cell model for gastro-intestinal experiments and have been employed in many studies [41].

It was found that the carotenoid cellular uptake did not vary significantly between the plum and cabbage varieties. The fractional carotenoid cellular uptake differed between the varieties, and carotenes were more bioavailable than xanthophyll. This is not what we expected, as xanthophylls, in mixed micelles, are found at the surface, while carotenes which are more hydrophobic are found in the core and are therefore more slowly liberated from the mixed micelles [42]. Interestingly, the cellular uptake of carotenoids was significantly higher in the coculture than in the monoculture. It was surprising that carotenoids were better taken up by the coculture than by the monoculture, as the HT-29-MTX cells are producing mucus, which could have acted as a barrier for their uptake. It is possible that the mucus aids in improving micelle binding to the cell surface via increasing the unstirred water-layer area, thereby facilitating carotenoid uptake. As the bioaccessibility of the polyphenols was

already impacted by the cream, the cellular uptake was also very low and could not reliably be measured by our methods.

Based on these findings, Kale and Plum 620 were the most promising varieties in delivering carotenoids to the gut in our studies, with 1.4 % (156 µg/100g) and 1.4 % (7.2 µg/100g) cellular uptake.

As the large intestine with its microbiota is known to potentially influence polyphenol and possibly carotenoid profile, we also attempted to estimate the influence of the colon. During colonic fermentation, polyphenols are further metabolized and new metabolites are produced, which may in part be more bioactive than their parent molecule [43]. In this study, the recovery of total carotenoids was between and 4 and 25%, the native polyphenols were not detectable as we already observed a loss of polyphenols after *in vitro* digestion. Our carotenoid recovery was in line with two other studies, one done by Serrano *et al.*, were the total recovery of carotenoids from spinach was 2 % -11 % [28] and a study done by Goni *et al.*, with fruit and vegetables showing a recovery of carotenoids between 0.6 and 12.5 % [44]. We have to state that we did not investigate the polyphenol metabolites, which could have been produced during colonic fermentation, as we did not have the corresponding standards and no mass spectrometry method available. Another limitation is that the colonic fermentation was done with pig faeces, whose microflora shows some differences to the human microflora, but this fermentation model was also used in other studies [45], and also human microflora differs substantially between subjects. A negative point of using pig faeces was that already our blank samples contained carotenoids (mainly zeaxanthin), which resulted from the diet (corn), and had to

be subtracted, further adding to the variability. However, the recovery of carotenoids following fermentation indicated that these may be potentially available in the colon, and also that a large fraction must react to unknown, possibly more polar degradation products. As it is not known [46] whether carotenoids can be taken up from the colon, this is a prominent gap in our knowledge that should be addressed in the future.

Anti-inflammatory aspects of plum and cabbage digesta

The next step after bioaccessibility and cellular uptake was to determine the effect of our different digests on selected inflammatory markers, cellular signalling cascades as well as their impact on the proteome of our different cell lines. To determine this impact we included in our coculture cell model system (Caco-2/HT29-MTX cells), THP-1 cells, which are often used as a cell model for immune modulatory studies. THP-1 cells are monocytes that can be differentiated into macrophages, which play an important role at the site of infection. Macrophages are responsible for antigen presentation, phagocytosis and of the production of cytokines [47]. The two different cell models, Caco-2 monoculture and the triple culture composed of the Caco-2/HT29-MTX coculture in the apical compartment and the THP-1 cells in the basolateral compartment, were stimulated for 24 h with LPS, IL-1β and TNF-α, after 6 h the Caco-2 monoculture and the coculture part of the triple culture were also treated with digesta. After 24 h, we measured the levels of IL-8, IL-6, PGE-2 and NO secretion, the mRNA expression of iNOS, COX-

2, NF-κB, Nrf-2, IL-8, IL-6, and MAPK as well as the Nrf-2 nucleus accumulation via immunostaining. These inflammatory markers have been chosen as they play an important role in IBD [48]. The results showed that in the monoculture and in the coculture part of the triple culture, the plum varieties significantly reduced the IL-8 secretion. The plum varieties Ersinger and Italian plum also significantly decreased the Nrf-2 mRNA expression and Duchy, Kale, Plum 620 and Ersinger also decreased significantly the Nrf-2 nucleus accumulation. In the THP-1 cells of the triple culture, Scots Kale and Kalorama significantly decreased IL-6 secretion as well as NF-κB mRNA expression. Thus, as with bioaccessibility, a large variability was seen between the different varieties, though not generally between plums and cabbages. To a certain extent, reduced IL-8 secretion was related to reduced NF-κB expression, which has been termed a "master switch" involved in many inflammation-related diseases.

Overall, the coculture showed a greater response than the monoculture. This could be due to the higher bioavailability of carotenoids of the coculture than of the monoculture, but it remains hypothetical

It has to be stated that our chosen inflammatory stimulus with IL-1β, TNF-α and LPS significantly increased the inflammatory response of IL-8, IL6, and NF-κB, but that there was no significant effect on Nrf-2, NO and PGE-2. We have chosen this inflammatory stimulus because it has been shown in earlier trials that this inflammatory stimulus can enhance the secretion of IL-8, PGE-2, NO as well as the activation of NF-κB [49, 50]. The addition of empty digesta also enhanced the inflammatory response, which is probably due to the

negative effect of the bile salts. The results thus allowed to conclude that while NF-κB was affected due to the inflammatory stimuli and partly reduced by the various treatments, these stimuli did not cause oxidative stress (Nrf-2) or COX-2 related responses. Though a relation between inflammation and oxidative stress has been shown [51], perhaps duration of exposure has been too short to cause such an effect in our model.

NF-κB is a transcription factor that is involved in inflammation and plays an important role in the upregulation of downstream genes, which then lead to the secretion of different cytokines and chemokines, e.g. IL-6 and IL-8. In the present study, at least in THP-1 cells, the digests reduced IL-6 secretion as well as NF-κB mRNA expression, being in line with the hypothesis that IL-6 is regulated via NF-κB. The plum varieties decreased the IL-8 secretion in the mono- and coculture. However, there was no measurable impact on IL-6/IL-8 mRNA expression, this could have been due to a transient activation only. It has also to be stated that the digesta was only put on the coculture part in the apical compartment, and the incubation time might have been too short to provoke an upregulation in the THP-1 cells. Another point is that in our triple culture, the THP-1 cells were not in direct contact with the other cells, which could also be the reason why the response of the immune cell culture (THP-1 cells) was low compared to the coculture. However, the inclusion of the THP-1 cells in our cell model is, however, a novel aspect and mimics more realistically gut behaviour, constituting a more advanced model of IBD.

Nrf-2 is another transcription factor that plays an important role in maintaining the physiological concentration of ROS. Interestingly, Nrf-2 nucleus accumulation was found to be reduced following the exposure of certain varieties (both cabbage and plums), which suggests that the added varieties rather reduced overall oxidant status, requiring less activation of further downstream antioxidant enzymes such as SOD, CAT, GPx, and HO-1. This is in contrast in part to higher individual doses of e.g. carotenoids, which have shown to enhance Nrf-2, possibly exerting a "positive form of oxidative stress", which is able to boost the bodies' own antioxidant system.

In our study, we used cabbage and plum varieties, thus a natural matrix, and with physiological concentrations of carotenoids and polyphenols. Overall, the results suggested that the cabbage and plum varieties only had limited effects on the reduction of inflammation and oxidative stress. However, exposure was only short-term, and we did not investigate the effect of colonic digested samples on anti-inflammatory or anti-oxidant effects, thus differing effects *in vivo* cannot be excluded. In a study done with berries subjected to *in vitro* digestion and colonic fermentation, it was shown that the berry fermented extract showed the same inhibitory effect on DNA damage and mutations in HT-29 cells than the berry *in vitro* extracts not subjected to colonic fermentation [52]. Another study was done with the human normal colon fibroblast cells CCD18-Co and ellagic acid and its gut microbiota-derived metabolites urolithins. They showed that the urolithins were able to reduce PGE-2 levels as well as NF-κB activation and that there was no effect of ellagic acid [53]

Furthermore, the cabbage and plum varieties that showed some impact on the different inflammatory markers, included, however, varieties with low concentrations of polyphenols and/or carotenoids, thus it is unlikely that the present effects are due to only carotenoids or polyphenols. Other available phytochemicals such as vitamin C or in cabbages also glucosinolates could have played a role in the reduction of the inflammatory markers. Again, we have to state that we did not measure the glucosinolate derivatives isothiocyanates that are an anti-oxidant component in *Brassicaceae*. It has been shown by Brunelli *et al.*, that glucomoringin derived isothiocyanates were able to reduce NF-κB activity by 70% in RAW-NF-κB cells [54]. Another study showed that berteroin decreased iNOS and COX-2 mRNA expression and through this also NO and PGE-2 secretion in LPS-induced raw 264.7 macrophages [55]. Jiang *et al.* also studied the effect of indol-3-carbinol (I3C) on THP-1 cells and showed that I3C reduced IL-6, IL-1β and NO secretion [56]. However, we investigated the vitamin C content as this is also an important anti-oxidant molecule, and we also measured the dietary fiber content.

Effects on the proteome

In this study, we were also interested in the impact of our digests on the proteome level of our different cell models. We have chosen to take for the proteomic study the cabbage and plum variety with the highest carotenoid and polyphenol content, *i.e.* Kale and Italian plum, and compared it with "empty digests", *i.e.* digesta without any plum or cabbage. Proteomic studies generally present a non-targeted approach

to study on a more broad level changes following e.g. certain interventions. Earlier studies had suggested [57] that carotenoids may influence proteins involved in oxidative stress, though, to our knowledge, no proteomic studies regarding the effects of carotenoids are available for models of IBD.

The study revealed that in the monoculture, 68 protein spots were differentially expressed due to Kale or Italian plum digesta. From these 68 protein spots, 27 were uniquely identifiable. In the coculture part of the triple culture, 206 protein spots were differentially expressed, from which 76 were uniquely identifiable. In the THP-1 cells alone, only 3 proteins were differentially expressed due to the Kale and Italian plum digesta, and only one protein was uniquely identifiable. Thus, the coculture showed again the highest reaction in comparison with the monoculture. This could be explained by the higher bioavailability of the carotenoids in the coculture.

Concerning differences between the kale and the plum, the Kale digesta also showed a higher impact on the proteome of Caco-2 cells as well as on the coculture, which may be explained by the higher amount of carotenoids in the Kale compared to the plum, though this remains speculative. It has also to be stated that Kale also contains other phytochemicals such as glucosinolates, which could also have contributed to the observed effects. The reaction of the THP-1 cells was very limited and again this might be explained by the short incubation time (18h) and the fact that the THP-1 cells were not in close contact with the enterocyte cells.

Most of the proteins differentially expressed in both cell models were down-regulated (56% for monoculture and 79 % for coculture). Most of the differentially expressed proteins were enzymes involved in antioxidant defence mechanisms, such as catalase, superoxide dismutase, glutathione S-transferase and possibly peroxiredoxin-4. All of these enzymes were down-regulated either in the monoculture or the coculture or in both by the Kale treatment. These findings could thus lead to the hypothesis that the carotenoids and polyphenols in the Kale digesta acted rather as anti-oxidants on the co-culture, decreasing the general redox and inflammatory status in the cells. The same was already observed in the transcriptomic study, where Kale decreased the Nrf-2 nucleus accumulation in co-culture, an important transcription factor in maintaining homeostasis of the redox state.

As already stated, this effect could also be due to other phytochemicals present in the Kale, which could activate other signalling cascades that we did not investigate. The glucosinolates derivatives, for example, are able to activate the extracellular signal-regulated kinase ERK, c-JUN N-terminal kinase (JNK), but they can also influence the production of antioxidants via direct activation of the aryl-hydrocarbon receptor (AhR) or via increasing the affinity of the AhR to xenobiotic response elements of antioxidants genes [19].

It has however also to be mentioned that during this study a lot of other catalytic activities (i.e citric acid cycle) were down-regulated, so it could also be possible that the Kale and plum treatment led to a general slowing down of cellular metabolism.

To conclude, we can say that some of the chosen plum and cabbage varieties were able to reduce certain inflammatory markers and were involved in altering cellular signalling cascades, however, there was neither general difference between plum or cabbage families, nor to carotenoid or polyphenol rich patterns. The plum and cabbage varieties that showed some inflammation-reducing effect included varieties with low carotenoid and polyphenol content, and this may be further in line with studies suggesting that these compounds do play a role especially regarding synergistic/additive effects with the food matrix and with other phytochemicals or micronutrients such as vitamin C, E [58, 59]. This work has thus allowed to yield further insights on the bioaccessibility and bioactivity of plant food constituents, has introduced a novel model to study their effects on the gut epithelium, strengthening recommendations toward the intake of whole fruit and vegetable consumption as promising vehicles to deliver anti-inflammatory and anti-oxidant active effects to the gut. However, more investigations are needed on the human side to confirm whether polyphenol and carotenoid-rich fruits and vegetables truly can aid in the prevention of IBD, or may alternatively ameliorate the severity of these diseases.

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9. Perspectives (Chapter 9):

Inflammatory bowel disease, primarily including ulcerative colitis (UC) and Crohn's disease (CD) are affecting more than 300 per 100 000 individuals in Western population [1]. At the moment, there is no curative therapy available, while current therapy has a focus on suppressing inflammation, but has only limited success and often results in adverse effects. Therefore, it is of great interest to find alternative strategies. A potential approach could be via preventive and adjuvant strategies, including diet, by eating vegetables and fruits with a high content of anti-oxidant molecules.

In this study, we showed that certain plum and cabbage varieties were able to reduce some of the inflammatory markers investigated and also had some impact on the signalling cascades investigated. However, during this study, new questions have been raised that may drive future research investigations.

Potential further *in vitro* investigations may include the following:

- The *in vitro* gastro-intestinal digestion which was used is a frequently employed and representative model, however, further improvements are possible: additional cells that are also present in the intestinal lining, such as M cells, could also be employed.

  We used a static *in vitro* gastro-intestinal digestion model, but there exists also already dynamic models that stimulate the dynamic conditions in the lumen of the gastro-intestinal tract [2]. However, these are quite expensive and time consuming to use, as

only 1 sample can be studied at a time, and also larger volumes of samples are required.

Furthermore, as a vehicle for carotenoids we used as a lipid source for the carotenoid micellarization cream, which appeared to have some drawbacks on polyphenol bioaccessibility. To improve also polyphenol bioaccessibility and to not impact carotenoid bioaccessibility, oil could be chosen as lipid source. These aspects show that also *in vivo*, it is important in what matrix, and in what combinations phytochemical rich food items are presented in order to maximise bioavailability aspects.

- Another important point is that polyphenols and carotenoids are metabolized during colonic fermentation, and that we did not investigate any potential metabolites. It has been shown by several studies that the metabolites are sometimes more bioactive then their parental molecules [3, 4]. The colonic fermentation seems to play a major role in polyphenol metabolism and should also be further investigated, however there are not many polyphenol metabolite standards available, which makes the investigation of these compounds difficult. Thus, in the future, it should be strived to better integrate these colonic aspects into studies investigating phytochemical bioactivity in *in vitro* studies.
- For the inflammatory part of this study we used a more sophisticated cell model as we also included an immune cell line, the THP-1 cells. This cell model could however still be further improved, in our cell model the Caco-2/HT-29 MTX cell model was on the apical side of the insert while the THP-1 cells were

cultivated on the bottom of a 6 well plate and were therefore not in close contact with the enterocyte like cells. An improvement could be done by cultivating the THP-1 cells on the basolateral side of the inserts or by employing 3D cell models [5]. Another interesting model could be the recently developed HuMix (human-microbial crosstalk) model, which allows the coculturing of microbiota and human cell lines under gastro-intestinal conditions [6], though being limited to study very small amounts of digesta.

- We used digesta of our plum and cabbage varieties, which included a large array of different phytochemicals. The plum and cabbage varieties that showed some responses, included also varieties with low carotenoid and/or polyphenol content. Therefore, we cannot say that the responses are due to carotenoids or polyphenols alone. To verify whether the response is due to the carotenoids and/or the polyphenols, one possible way would be to reconstitute the carotenoid and polyphenol content of our digesta and to evaluate then: (i) the impact of carotenoids, (ii) the impact of polyphenols and (iii) the synergistic/additive effect of carotenoids and polyphenols. However, this is impeded by the fact that re-constitution of a full profile is very difficult, expensive, and still incomplete as the binding to the matrix and their cellular locations cannot be mimicked, but are expected to also play a role, again, via affecting bioaccessibility.
- Another important point is the factor time. We investigated the impact of inflammatory stimuli and of the digesta on the enterocyte cells for 24 hours. However inflammation in inflammatory bowel disease is a chronic state with some acute

inflammation phase but also a chronic and systemic aspects, and therefore a study over longer durations would be of advantage. We also have measured the different inflammatory markers only after 24 hours and could have missed the maximum secretion of these selected inflammatory markers. Another point is that the duration of incubation with the digesta (16 hours) may not have been long enough for the phytochemicals to reach the THP-1 cells and to provoke there a full response. However, we did not dare to increase the incubation time of the digesta on the cell culture because we would then have increased the risk of microbiological contamination. For this reason, and to study more long-term effects, the best approach would be to continue with *in vivo* experiments, once promising food items have been singled out.

- We also have to state that for practical reasons we used for our study Caco-2 and HT-29 cells with slightly varying number of cell passages (i.e. 70-100 for Caco-2 cells). This could have had an impact on the response of the cells to the different digesta and inflammatory stimuli exposure. The optimal approach would be to use always the same passage number range for all experiments, even if this is more time-consuming as new cells need to be defrozen and cultured until the cell passage number needed.

Further *in vivo* experiments:

There exist already a large number of animal models to investigate the impact of carotenoids and polyphenols on inflammation [7]. However animals have not the same metabolism as humans, differ in their microbiota, but could already together with the *in vitro* testings give an approximatively direction on how carotenoids and polyphenols act

in humans [8]. The colonic fermentation, which we investigated was done with pig faeces, which certainly differ in their microbiota to humans, but also in humans the microbiota is changing from one person to another. Some studies have already been conducted in humans, with individual carotenoids and polyphenols but also with complex matrices [9, 10]. However not many studies have investigated the impact of carotenoids and polyphenols on the inflammatory status of the intestine in patients with UC and CD [11]. Nevertheless, the fact that at least polyphenols may in part act as prebiotics should not be overlooked and also be considered in the future.

Human studies are difficult to establish, because studies with individual compounds, in form of supplements, are easy to handle but often show no or negative effects, as synergistic effects with other micronutrients cannot take place and the bioactive compounds are often given in too high concentrations. On the other side, human studies with complex matrices such as fruits and vegetables show some health beneficial effects, but then this effect cannot be attributed to one bioactive compound, as here the synergistic/additive effects might play a role. Another important factor, which should also be taken into account is the health state of the persons used for such studies. Healthy people often have different responses than people having health problems, including for example people with IBD. This may, for example, result in practical limitations of what people can ingest, as IBD subjects may be more prone to ruther gas production and pain resulting from the intake of fibre-rich products [12].

The WHO is recommending to eat 400 g of fruits and vegetables each day to prevent chronic diseases. Until today, there are no health claims

available for carotenoids related for either anti-inflammatory properties, or for polyphenols. Most of the claims have been rejected because studies were done with whole fruits and vegetables and their positive impact on some chronic diseases could therefore not be attributed to a single phytochemical.

There are also some further activities currently ongoing, such as the EU COST action EUROCAROTEN, where the idea is to provide further details of how carotenoids can impact various health aspects, and also the formation of a European network on carotenoids is targeted. There is also another COST action of somewhat broader scope, targeting the inter-individual physiological response and bioavailability of bioactive compounds of plant foods in relation to cardio-metabolic endpoints (COST action POSITIVe).

Future activities targeting fruit and vegetable interventions related to chronic disease prevention are much desired, including IBD.

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